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DETECTION AND ISOLATION OF, AND PROTECTIVE IMMUNISATION AGAINST, THE PHOMOPSIN MYCOTOXINS

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(57) Claim

1. Antibodies specific to the phomopsin mycotoxins of the fungus *Phomopsis leptostromiformis*.

4. A phomopsin-macromolecule carrier conjugate.

7. A method for the production of antibodies specific to the phomopsin mycotoxins of the fungus *Phomopsis leptostromiformis*, which comprises immunising an animal with a phomopsin-macromolecule carrier conjugate according to any one of claims 4 to 6, and recovering antibody-containing serum from the animal.

10. A vaccine composition for stimulating protection in animals against lupinosis, which comprises an effective amount of a phomopsin-macromolecule carrier conjugate according to any one of claims 4 to 6 as an active immunogen, together with an acceptable carrier or diluent therefor.

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COMPLETE SPECIFICATION FOR THE INVENTION ENTITLED:

"DETECTION AND ISOLATION OF, AND PROTECTIVE IMMUNISATION AGAINST, PHOMOPSIN MYCOTOXINS"

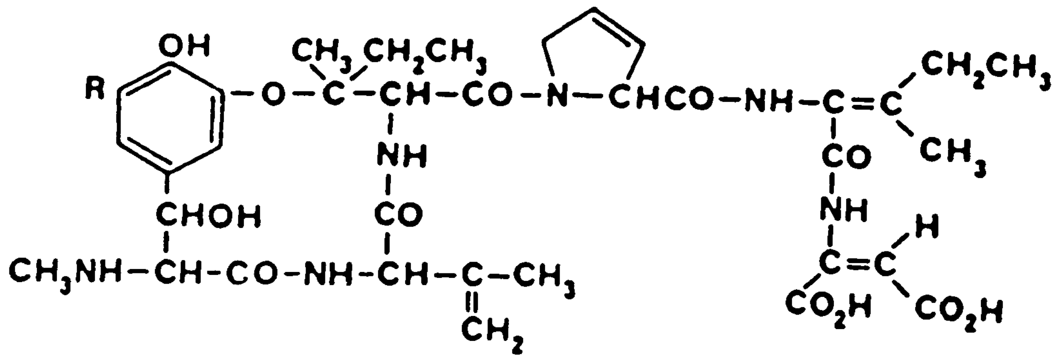
The following statement is a full description of this invention, including the best method of performing it known to us:

DETECTION AND ISOLATION OF, AND PROTECTIVE IMMUNISATION AGAINST, PHOMOPSIN MYCOTOXINS

This invention relates to the preparation of phomopsin-macromolecule carrier conjugates, phomopsin-specific antibodies and phomopsin-labelled reagents (e.g. phomopsin-enzyme conjugates, etc.) and the use of these to detect, quantitate and isolate the phomopsin mycotoxins. The phomopsin-macromolecule carrier conjugates are also of use in immunising animals against the disease lupinosis, a liver disease of sheep, cattle and horses which is caused by ingestion of the phomopsin mycotoxins.

The phomopsin mycotoxins are a group of highly toxic, natural products exerting their effect by binding to tubulin and inhibiting formation of microtubules, cytoskeletal structures involved in a number of vital cellular processes including cell division. The phomopsins are produced by the fungus *Phomopsis leptostromiformis* which is a specific pathogen of *Lupinus* spp. (lupins) but which can grow readily on other substrates such as maize. Other *Phomopsis* species are also potential sources of these toxins. They are common pathogens of a variety of food crops including soybean, potatoes, tomatoes and fruits such as avocados, peaches etc. Phomopsin A (PhA), the main mycotoxin, and phomopsin B (PhB) are linear hexapeptides containing a thirteen membered ether ring, see Formula I below, (Edgar et.al., 1986; Mackay et.al., 1986). A third toxin,

phomopsin C (PhC), has been isolated but its structure is not yet fully determined.



Formula I. Structure of the phomopsins

Phomopsin A R = Cl

Phomopsin B R = H

Lupins are grown as a grain legume crop and their seeds are used in animal feed and increasingly in items of human diet. Lupins also promote soil fertility and they are therefore grown in rotation with cereal crops. The lupin stubble and fallen seed left after harvesting are highly nutritious and can provide beneficial feed for grazing animals.

Because of the risk of the disease lupinosis associated with lupin seeds and the products derived from them, regulations in Australia require a maximum contamination of 5 p.p.b. (5 mg/kg) of the phomopsin mycotoxins in food for human consumption (Priestly, 1986). A similar acceptable-level of contamination by these mycotoxins is likely to be established in other countries either growing or importing lupin seed or lupin containing products.

On-farm production losses resulting from the disease lupinosis and under-utilisation of lupin seed and stubble due to fear of lupinosis are considerable (Culvenor, 1985), so that, as well as the requirement for a practical assay for phomopsins in human food, farmers

using lupin stubble and seed as animal feed also need a means of determining the amount of phomopsin present and the degree of hazard posed by particular stubbles and seed lots before exposing animals to them. Commercial livestock feed producers incorporating lupin seed into their products will also benefit from the immunoassay which forms a part of this invention.

Currently available methods of assaying the phomopsin mycotoxins have a number of drawbacks especially with regard to time taken per assay, technical skills required to perform the assays, degree of sample purification needed, cost, sensitivity and specificity (Hancock *et.al.*, 1986).

The laboratory detection of the phomopsins by most biological and all chemical techniques requires their prior extraction from the toxic material. This can be accomplished by steeping the toxic seed or stubble in aqueous:alcohol mixtures or aqueous buffers at pH 8-9. It is often necessary to further purify these extracts for chemical detection and for some methods of biological detection. A number of techniques for the purification of phomopsins are available, including solvent partitioning, adsorption chromatography and ion exchange chromatography (Hancock *et.al.*, 1986).

The antimitotic effect of the phomopsins has been the basis of a number of bioassays, the most reliable and sensitive method being a nursling rat bioassay using 14-day old rats (Peterson, 1978). The method involves intraperitoneal injection of phomopsin extracts at the rate of 1 ml/100g body weight. Eighteen hours later the animals are killed and liver sections prepared, examined microscopically and scored for abnormal mitotic figures. Five rats are used at each dose level of a twofold dilution series and a group of control rats receive

saline or a toxin reference sample. A large number of animals are required for a single assay and several days of technically skilled work are involved in assessing the results. The cost of this type of assay is therefore prohibitive except as a research tool. It is also very time consuming and not suitable for routine quality control of food products etc for phomopsin mycotoxin contamination.

Of the physico-chemical methods which have been employed, high performance liquid chromatography (HPLC) has been used most extensively (Hancock et.al., 1987). Sample through-put however is reported to be four samples in two days and the limit of detection is 200mg of mycotoxins/kg of seed and 500 mg/kg of lupin stubble (Hancock, et.al, 1986). The phomopsins are detected by UV absorption at 280 nm and UV absorbing impurities with similar retention times to those of the phomopsins can interfere with the HPLC assay, reducing both specificity and sensitivity of the assay.

Immunoassays have been developed for a number of mycotoxins (Chu, 1984). They offer several advantages over other methods especially where quality-control is concerned. Sensitivity and specificity is usually high, minimal clean-up of samples is normally required, sample throughput is rapid and cost per assay and equipment costs are low. However, prior to the present invention, attempts to produce such an assay for the phomopsins had been unsuccessful (Hancock et.al., 1986).

In accordance with a first aspect of the present invention there are provided anti-phomopsin antibodies, that is, antibodies specific to the phomopsin mycotoxins of the fungus *Phomopsis leptostromiformis*, which may be monoclonal or polyclonal. This invention also includes a method for producing such antibodies by immunising an

animal with a phomopsin-macromolecule carrier conjugate and recovering antibody-containing serum from the animal.

In another aspect, the present invention relates to a method for the detection, and optionally quantitation, of phomopsin mycotoxins in a sample which is characterised by the use of anti-phomopsin antibodies in an immunoassay.

The present invention also extends to a vaccine for stimulating protection in animals against lupinosis. Such a vaccine would allow protected animals to feed with impunity on phomopsin-contaminated lupin seed and stubble and reduce production losses resulting from lupinosis and under- utilisation of lupins from fear of the disease.

In this aspect, the invention provides a vaccine composition for protective immunisation of an animal against lupinosis, which comprises a phomopsin-macromolecule carrier conjugate as an active immunogen. The invention also extends to a method for protective immunisation of an animal against lupinosis, which comprises administration to the animal of a vaccine composition comprising a phomopsin-macromolecule carrier conjugate as an active immunogen.

In yet another aspect, the present invention comprises a method for isolation of phomopsin mycotoxins from a sample, which comprises contacting the sample with immobilised anti-phomopsin antibodies.

The phomopsin mycotoxins have a role to play as tools in biological research. They are amongst a small group of chemically diverse substances known to act as microtubule inhibitors by binding to specific sites on microtubular sub-unit proteins (tubulins). These

antimicrotubule substances are of increasing biological interest and are important in a number of areas of chemotherapy, including use as anticancer agents, anthelmintics and herbicides. They have also been used extensively in providing an understanding of the fundamental biochemistry of tubulin and microtubules much of which remains unknown (Dustin, 1984). The phomopsins are a valuable new addition to this group of antimicrotubule agents (Lacey *et.al.*, 1986). Unlike the other agents of this type, they are quasipeptide in nature and as such may be conformation-constrained mimics of endogenous peptides involved in the natural modulation of microtubule assembly-disassembly. Recent research has shown that the phomopsins are the most effective of all the antimicrotubule agents known (Lacey, *et.al.*, 1986).

The phomopsin-specific antibodies of this invention will be useful in purifying the phomopsins as fine chemicals for use in research aimed at achieving a greater understanding of microtubule biochemistry and associated phenomena. The other reagents and methods of this invention will also aid such research by providing means of detecting and quantitating the phomopsins and identifying phomopsin-binding proteins *in vivo* and *in vitro*.

In addition, the phomopsin-specific antibodies of this invention may be used in the preparation of affinity-purified phomopsins for use in the production of phomopsin-macromolecule carrier conjugates as disclosed herein.

As broadly described above, the present invention pertains to chemical synthesis and immunochemical production of reagents useful in detecting, isolating and quantitating the phomopsin mycotoxins and in protectively immunising animals against their toxic effects. The

assays and detection methods employ phomopsin-labelled reagents and anti-phomopsin antibodies which react specifically with the phomopsin mycotoxins. The latter are also employed in affinity purification of the phomopsins while protective immunisation involves vaccination with phomopsin-macromolecular carrier conjugates.

Anti-phomopsin antibodies.

The phomopsins have low molecular weights and are not antigenic. Polyclonal or monoclonal antibodies are therefore produced after attaching (conjugating) naturally occurring mixtures of these mycotoxins to suitable carrier macromolecules such as haemocyanin (particularly keyhole limpet haemocyanin), fetuin, ovalbumin and bovine serum albumin. Other suitable carriers include, for example, tetanus toxoid and diphtheria toxoid. In each case a variety of attachment procedures can be used involving for example carbodiimide reagents, N-hydroxysuccinimide and mixed anhydride methods of conjugation to form amide and ester or other covalent links with the carrier macromolecules. The phomopsins can be attached directly to the carrier or through a linking structure.

Immunisation, using phomopsin conjugates, can be performed by a variety of different protocols. In general the conjugates are administered to experimental animals by a primary injection of an aqueous emulsion with Freund's complete or incomplete adjuvant, or other adjuvants such as alum precipitates or DEAE dextran, followed by one or more boosting injections using the same adjuvants. During and after the immunisation procedure, sera taken from the animals may be screened for production of specific antibodies to the phomopsin mycotoxins using phomopsin-labelled reagents described below.

The anti-phomopsin antibodies needed for isolating and assaying phomopsins can be conveniently produced in large quantities using sheep. The anti-phomopsin antisera can be used in the assay procedures and isolation methods either directly or after purification.

Monoclonal anti-phomopsin antibodies may be prepared by methods which are well known in the art, again using phomopsin-macromolecule carrier conjugates as the primary immunising agent.

Phomopsin-labelled reagents.

Phomopsin-labelled reagents are produced using an enzyme, a radioactive material (e.g. ^{14}C, ^{125}I), an optical label such as a fluorescent material or with some other easily detected entity (e.g. biotin) as a tracer.

Immunoassays.

The immunoassays of this invention include competitive binding assays and "sandwich" assays. They provide rapid, highly sensitive, specific methods of detecting and quantifying the naturally occurring phomopsin mycotoxins. For instance, a solid-phase competitive enzyme linked immunosorbent (ELISA) assay may be performed in one and a half to four hours, depending on the accuracy required, and is capable of detecting and quantitating levels of phomopsin mycotoxins as low as 0.1 ng/ml (range 0.1-200 ng/ml). The sandwich assay is less sensitive but provides a convenient and rapid means of screening hybridomas and sera for anti-phomopsin antibodies.

Phomopsins can be extracted from samples with, for example, methanol/water (4:1); the filtered extracts, diluted in an assay buffer, are suitable for analysis. In a typical format of the assay, phomopsin standards and diluted extract aliquots in assay buffer are incubated

with a phomopsin-enzyme conjugate in the wells of microtitre trays coated with the anti-phomopsin antiserum and then, after washing, incubated with a substrate which is converted by the enzyme into a coloured product. After a predetermined time to allow the color to develop, a standard curve is constructed by plotting the optical density of the color in the wells against the amount of standard phomopsin added. This curve is then used to determine the amount of phomopsin in the unknown samples.

The immunoassays of this invention can be used to detect and quantify phomopsin mycotoxins in a liquid sample. As well as extracts of foodstuffs, the liquid samples may include essentially all biological fluids such as blood or components of blood such as plasma or serum and urine, lymph, extracts of tissues, lupin seed, lupin stubble, etc. The liquid samples may also be extracts or supernatants of microbial cultures including both laboratory and naturally occurring microbial cultures (e.g. rumen fluid).

In the solid phase assays and affinity purification methods of this invention, the anti-phomopsin antibodies are preferably immobilised by affixing them to a variety of solid phases. Well-known solid phases include ELISA trays and beads formed from glass, polystyrene, polypropylene, dextran and other materials; tubes formed from or coated with such materials etc. The antibodies can be either covalently or non-covalently bound to the solid phase, by techniques such as covalent bonding via an amide or ester linkage or adsorption. Those skilled in the art will know many other suitable solid-phases and methods of immobilising antibodies thereon, or will be able to ascertain such using no more than routine experimentation.

Immunopurification of the phomopsin mycotoxins may be performed according to the following procedure. Antibodies which bind the phomopsins are immobilised by affixing them to a solid phase (see above) to form an immunoadsorbent which specifically adsorbs the phomopsin mycotoxins. A liquid sample from which the phomopsins are to be isolated is contacted with the immunoadsorbent under conditions which allow the mycotoxins in the liquid to be adsorbed by the immunoabsorbent. The immunoadsorbent and the liquid are then separated. Usually, the immunoadsorbent is washed, and then the mycotoxins are recovered from the immunoabsorbent.

In conventional affinity chromatography, the immunoadsorbent usually comprises antibody-conjugated particles which are packed into a column. This is the convenient embodiment for phomopsin mycotoxin affinity purification. Anti-phomopsin antibody-conjugated particles, preferably beads, are packed into a column and the phomopsin mycotoxin-containing liquid is passed through the column. The phomopsins are retained because of the binding affinity of the immunoadsorbent for the mycotoxins. After washing, the phomopsins are recovered, most often by elution with an eluant which causes the bound mycotoxins to dissociate from the immunoadsorbent.

The invention is illustrated further by the following examples, and in the accompanying drawings, in which:

Figure 1 shows the effect of buffer, pH and temperature on coating of microtitre trays with antiserum (1/10,000 dilution). Values are means of four determinations. Optical density was measured 45 min. (*22.5 min.) after the addition of enzyme substrate.

Figure 2 shows the effect of different washing solutions in EIA. Microtitre trays were coated with anti-phomopsin antiserum (1/10,000 dilution) in PBS, pH 5.0 or carbonate buffer, pH 9.6 using various washing solutions. Washing solutions were:

○——○ 0.05% tween 20 and 0.1% gelatine in PBS

△——△ 0.05% tween 20 in 0.15M NaCl

□——□ 0.05% tween 20 in water.

Figure 3 shows the effect of different incubation times in the EIA. Microtitre trays were sensitised with anti-phomopsin antiserum (1/10,000 dilution) in carbonate buffer (pH 9.6), overnight at 37°C. Free phomopsin and enzyme-labelled phomopsin were added and the mixture incubated for various times prior to washing, adding substrate and measuring optical density. Results are plotted in terms of (A) optical densities and (B) the proportion of enzyme-labelled phomopsin bound (B/B_o x 100).

Figure 4 shows a typical displacement curve of enzyme-labelled phomopsin by added free phompsins at a range of standard concentrations. The data was generated using microtitre trays coated with 1/10,000 dilution of CAR-HAEM anti-phomopsin antiserum in carbonate buffer, pH 9.6 and a 1/200 dilution of phomopsin-horseradish peroxidase stock solution was used as the detecting agent. The graph shows reducing enzymic activity (decreasing optical density) in wells due to displacement of the phomopsin-horseradish peroxidase conjugate with increasing amounts of added free phomopsins. Values are the mean ± standard deviation of 6 determinations.

Figure 5 shows antibody titres of 10 sheep vaccinated with phomopsin-keyhole limpet haemocyanin conjugate at the beginning, end and 6 months after the conclusion of the challenge with phomopsin mycotoxins.

Antibody titres remained essentially unchanged during the 4 week challenge.

Figure 6 shows mean residues of feed left by immunised and unimmunised sheep during challenge with phomopsins (50µ/kg body weight). Arrowheads indicate days of phomopsin administration. Inappetence is a sensitive sign of lupinosis.

Figure 7 shows changes to mean BSP clearance rates (a measure of liver function) during challenge with the phomopsins. BSP values are normally between 7 and 12. Unimmunised sheep showed significant liver dysfunction indicated by low BSP values. Three immunised sheep (Nos. 2, 7 and 8) with low antibody titres (Fig.5), also showed evidence of liver incapacity. The remaining 7 immunised animals gave normal BSP clearance values throughout the challenge.

Figure 8 shows mean serum aspartate aminotransferase (AST) and bilirubin levels (indicators of liver damage) in immunised and unimmunised sheep during challenge with the phomopsins compared with the value for unimmunised, unchallenged control sheep.

Figure 9 shows correlation of the antibody titres of 10 immunised sheep with liver function indicated by BSP clearance data. Three sheep (Nos. 2, 7 and 8) with low antibody titres (Fig.5) showed evidence of liver dysfunction during challenge with the phomopsins.

Figure 10 shows mean anti-phomopsin antibody titres at various stages of immunisation of sheep using the different immunogen formulations: phomopsin/fetuin; phomopsin/bovine serum albumin; phomopsin/haemocyanin; phomopsin/tetanus toxoid; phomopsin/diphtheria toxoid.

Figure 11 shows phomopsin standard curves of optical density, generated from plates coated with CAR-HAEM serum by the carbonate method compared with the glutaraldehyde pretreatment method.

Figure 12 shows optimisation of glutaraldehyde concentration for coating. Plates were pretreated with glutaraldehyde diluted in 0.1M carbonate buffer to 0.1, 0.2, 0.5, 1.0 and 2.0%, then assayed with phomopsin standards as described.

Figure 13 shows optimisation of coating antiserum for the glutaraldehyde coating method. CAR-HAEM antiserum was diluted 1/5,000-1/10,000 in 0.1M phosphate buffer for coating onto plates pretreated with 0.1% glutaraldehyde.

Figure 14 shows comparison of three phomopsin conjugates C_1, C_2 and C_3. Competition of each conjugate with the phomopsin standards is plotted in terms of (A) optical densities and (B) the proportion of conjugate bound (B/B_o x 100).

Figure 15 shows optical densities generated by different substrates compared with the ABTS reaction at 45 minutes: (A) OPD, stopped after 30 and 45 min.; (B) TMB, stopped at 30 min.

E X A M P L E S

EXAMPLE 1

Preparation and characterisation of phomopsin-protein conjugates.

A phomopsin-keyhole limpet haemocyanin conjugate (CAR-HAEM) was prepared as follows:

Keyhole limpet haemocyanin, 6.5 mg (MW 3,000,000 - 7,500,000) in 0.375 ml H_2O, pH 8.5 and 1.9 mg (9.91 µmol)

of 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide.HCl in 0.2 ml H_2O, pH 8.5 were added to 6.5mg (8.25 µmol) of mixed crystaline phomopsins from cultures of *Phomopsis* on lupin seed (Culvenor *et.al.*, 1977), dissolved in 50 µl of 1:2 dimethylformamide: dimethylsulphoxide. The mixture was stirred at room temperature for seven hours and then for a further 48 hours at 4°C in the dark.

After the reaction, free phomopsins were separated from the protein conjugate by ultrafiltration using a Centricon 30 microconcentrator (30,000 MW cutoff). The retentate was washed four times with 2 ml of H_2O, pH 8.5. After the final washing, the retentate was redissolved or resuspended in 2.6 ml saline pH 8.5 (2.5 mg protein/ml). An aliquot (0.1 ml) was diluted ten-fold and used to determine the amount of phomopsins attached to the carrier protein spectrophotometrically. The remaining CAR-HAEM conjugate was divided into aliquots of 0.4 ml/tube and stored at -18°C.

EXAMPLE 2

Preparation and characterisation of phomopsin-enzyme conjugates.

Phomopsin-horseradish peroxidase conjugates were prepared by a water-soluble carbodiimide method at pH 8.5.

Horseradish peroxidase 1 mg (0.025 µmol) in 0.075 ml of water, ph 8.5, and 1.65 mg (8.6 µmol) of 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide.HCl in 0.075 ml of water pH 8.5 were added to 0.079 mg (0.1 µmol) mixed crystalline phomopsins from cultures of *Phomopsis* on lupin seed (Culvenor *et.al.*, 1977). After three days at room temperature, free phomopsins were removed by ultrafiltration using a Centricon-10 microconcentrator (10,000 MW cutoff). The retentate was washed four times with 2 ml H_2O, pH 8.5. The degree of conjugation was

estimated from absorbance data at two wavelengths - 400 nm for horseradish peroxidase and 278 nm for the phomopsins. The retentate was further diluted to 1/9.33 and stored in 0.1 ml aliquots at -18°C. It was further diluted (1/62.5 to 1/200) before use.

By varying the phomopsin:enzyme molar ratio as set out in Table 1 below, conjugates with different phomopsin:enzyme ratios were synthesised.

<u>TABLE 1</u> Phomopsin-peroxidase conjugates produced using different enzyme:phomopsin ratios.

MOLAR RATIO (Phomopsin:enzyme: carbodiimide)	DEGREE OF CONJUGATION (Phomopsin:enzyme)
4 : 1 : 344	1.67 : 1
12 : 1 : 344	4.19 : 1
25 : 1 : 344	4.62 : 1

EXAMPLE 3

<u>Preparation of anti-phomopsin antisera</u>.

Anti-phomopsin antiserum was prepared by injection of CAR-HAEM conjugate into sheep.

The dose of phomopsin-protein conjugate injected into the sheep was calculated from the phomopsin:protein molar ratio. A range of 2.5-5 µg of phomopsins/kg body weight was chosen so that the total phomopsin dose was not more than a level known to produce very minimal toxic effects (Jago *et.al.*, 1982) and yet high enough to produce an antibody response. Each dose of conjugate was administered by subcutaneous injection over four sites as a 1:1 emulsion in Freund's complete adjuvant. Booster

injections, given as 1:1 emulsions in Freund's incomplete adjuvant, were administered at two to three week intervals for the first two boosters and at four to five week intervals thereafter.

Ten ml of blood was collected at regular intervals to monitor the appearance of phomopsin specific antibodies. Antisera were characterised in terms of phomopsin-binding capacity using ELISA trays to bind antibody and using a phomopsin-horseradish peroxidase conjugate as detecting agent.

Pre-immunisation sera from the sheep used did not bind phomopsin-horseradish peroxidase conjugates at any dilution tested (1/500, 1/1000, 1/5000) while sera from the same sheep two weeks after the second booster injection strongly bound the same phomopsin-horseradish peroxidase conjugates.

When high titres of anti-phomopsin antibodies were detected, sera were collected and stored in small aliquots at -76°C.

Binding of the phomopsin-enzyme conjugates to antiserum coated microtitre trays was shown to be through the attached phomopsin molecules since trays prepared with the same sera and treated with unconjugated horseradish peroxidase showed no residual enzymic activity after washing. In addition, there was clear evidence of competition for binding sites between enzyme-conjugated and free phomopsins at very low phomopsin levels (0.1 ng - 20 ng/ml) providing the basis for a very sensitive ELISA assay.

EXAMPLE 4

Competitive binding immunoassays for the phomopsins.

Antiserum was diluted (1/10,000) in phosphate buffered saline (PBS, Oxoid) or in carbonate buffer pH 9.6 with or without sodium azide (Saunders, 1979). Diluted antiserum (100 µl) was added to the wells of microtitre plates and these were incubated overnight at 4°C and 37°C. Figure 1 shows the effect of different buffer, pH and temperature on the coating of the microtitre plates.

Incubation and washing protocol (all at room temperature)

1. Plates were washed four times with 250 µl/well of wash solution (0.05% tween 20, 0.1% gelatin in PBS or 0.05% tween 20 in 0.15 M NaCl or 0.05% tween 20 in H_2O). During the washing procedure, the plate is turned upside down and the contents of the wells are shaken out by striking the plate firmly on a paper towel. Figure 2 shows the effect of the different washing solutions.

2. Aliquots (50 µl) of dilutions of phomopsin samples in assay buffer (0.1% gelatin or preferably 0.5% BSA in PBS, pH 8.2) or phomopsin standards in assay buffer and 50 µl of an appropriate dilution of the phomopsin-enzyme conjugate were added to each well to initiate a competitive binding process.

3. The plates were covered and incubated at room temperature. Presence or absence of phomopsins in the samples was readily detected by a ½ hour incubation. Accurate determination of phomopsin concentration in samples however required 2-3 hr

incubation. Figure 3 shows the effect of different incubation times.

4. After incubation, the plates were washed five times with wash solution.

5. The amount of phomopsin-enzyme conjugate bound to the wells of the ELISA plates was determined by the addition of 100 µl of substrate solution [1mM, 2,2'-azino-bis-(3-ethylbenzthiozoline sulphonic acid), 0.03% hydrogen peroxide in 0.1 M citric acid (pH 4.2)] to the wells.

6. After 45 minutes, the optical density at 414 nm was measured.

7. A standard curve was constructed by plotting optical density at 414 nm against the amount of authentic phomopsin added (Fig.4) and this was used to determine the amount of phomopsin in unknown samples.

EXAMPLE 5

Sandwich immunoassay for the phomopsins.

Two concentrations of a natural mixture of phomopsins (50 ng/well and 1µg/well) in 50µl of carbonate buffer pH 9.6 or in PBS buffer pH 8.2 were used to coat the wells of a microtitre plate. After overnight coating at 37°C, the plate was washed four times with washing solution (0.05% tween 20, in 0.15 M NaCl) and 100µl of a 1/5,000 dilution of sheep anti-phomopsin antiserum in assay buffer (0.1% gelatin in PBS, pH 8.2) was added and incubated for 3 hours at room temperature. The plate was again washed and 100µl of a 1/200 dilution of DAS-HRP [donkey anti-sheep IgG-horseradish peroxidase, Serotec] was added to the wells. After 2 hours incubation at room

temperature, the plate was washed four times and 100µl of substrate solution [1mM, 2,2'- azino-bis-(3-ethylbenzthiozoline sulphonic acid), 0.03% hydrogen peroxide in 0.1 M citric acid (pH 4.2)] was added to the wells. The results are shown in Table 2.

TABLE 2 Binding of DAS-HRP to sheep anti-phomopsin antiserum on a phomopsin-coated microtitre tray as indicated by enzymic activity in the wells.

Phomopsin/well	Enzymic activity in wells (optical density)	
	Carbonate buffer	PBS buffer
50ng	1.20	0.42
1mg	>2.00	1.06

Wells without phomopsin coating showed no enzymic activity nor did phomopsin coated wells treated with a 1/200 dilution of PH-HRP [phomopsin-horseradish peroxidase conjugate] used as a control enzyme conjugate.

Phomopsin coated microtitre trays can also be used to screen hybridoma culture fluid or sera of vaccination animals for anti-phomopsin antibodies using an appropriate enzyme-labelled second antibody.

EXAMPLE 6.

<u>Vaccination of Sheep against Lupinosis</u>

A. Sheep.

25 Border Leicester X Merino ewes were individually housed and fed 1000 g/day of a lucerne-oaten chaff mixture and had water available *ad.libertum*. The sheep were randomised into two groups of 10 and one group of 5 on the basis of body weight, bromosulphophthalein (BSP) clearance rates, serum bilirubin and AST (aspartate

aminotransferase) levels. All of these parameters were within normal limits for all sheep in the initial tests.

B. **Immunogen and immunisation protocol.**

The synthetic vaccine chosen for this experiment was a keyhole limpet haemocyanin conjugate prepared using carbodiimide as the coupling reagent (see Example 1). The 10 sheep in group 1 were immunised by subcutaneous injection of 1.0 mg of conjugate in a 1:1 emulsion in Freund's complete adjuvant, followed by 3 boosters of 0.5 mg of conjugate emulsified in Freund's incomplete adjuvant. Blood samples were taken at the time of each injection, at the commencement of and twice weekly during the challenge for monitoring of anti-phomopsin antibody titres.

C. **Measurement of anti-phomopsin antibody titres.**

Phomopsin dissolved in sodium bicarbonate buffer was bound to trays (50ng/well) by overnight incubation at 37°C. Trays were then washed in PBS-tween 20 and 100 µl of serial dilutions of serum were added. The trays were then incubated at 20 °C for 2 hours, washed and 100 µl of 1/1000 dilution of donkey anti-sheep IgG-HRP conjugate added. After a further 2 hours incubation, the plates were again washed and substrate solution added, the intensity of the developed colour being directly related to the antibody titre. Antibody titres are expressed as the reciprocal of the dilution giving an EIA reading equivalent to that of preimmune serum (at the same dilution) plus twice the standard deviation.

D. **Phomopsins.**

Phomopsins used for challenge were obtained from 4 crystalline batches prepared from *Phomopsis leptostromiformis* culture on moist, autoclaved lupin seeds. The toxin was dissolved in dilute sodium bicarbonate, and assayed by microbore HPLC with detection

by UV absorption spectra. All peaks showing characteristic phomopsin spectra were included in quantitation of phomopsin which was based on peak areas at 280 nm.

E. Challenge.

Both groups of 10 sheep were challenged with phomopsin administered orally (using a syringe and rubber tube) 5 days per week at the rate of 50 µg/kg/day. Doses were prepared each day by dilution in water of a nominal 1 mg/ml ethanolic solution the quantitation of which was as described above. The dose rate chosen was the same as that used by Peterson *et.al.* (1987) which caused the death of 3/3 sheep in 32 days (20 doses). Throughout the challenge period, a variety of clinical parameters were monitored as indicators of phomopsin damage. Daily food intake was measured for each animal, BSP clearance, serum AST and serum bilirubin levels were measured twice weekly (Mondays and Thursdays) and body weight was measured at the start mid-point and end of the challenge period. AST and bilirubin levels were determined using commercial assay kits (Sigma). BSP clearance was assessed by administering a standard dose of BSP *iv* in the jugular vein using a syringe and needle. Blood samples were taken at 1 and 10 minutes to give a "maximum" and cleared figure for BSP concentration and these figures used to calculate a clearance figure according to the formula:

$$K = \frac{\log(\text{BSP @ } 1') - \log(\text{BSP @ } 10')}{0.303 \text{ X Actual time difference}}$$

The K values are multiplied by a factor to give values in the range 0-12 with values between 7 and 12 being considered to be normal.

F. RESULTS.

1. Anti-phomopsin antibody titres.

Each of the 10 immunised sheep developed high titres of antibody, although there was a 7.5-fold spread of values (10,000 to 75,000) between maximum responses of individual animals. In the absence of further booster injections, the antibody titres declined steadily but slowly, to about half the maximum, throughout the duration of the challenge phase and over the ensuing 6 months (Figure 5).

2. Challenge.

(a) General. In general, the toxic effects of the phomopsin in the unimmunised group were slightly lower than that which had been aimed for. Using the same dose rate, Peterson *et.al.* (1987) achieved 100% mortality in 32 days (20 doses of 50 μg/kg over 4 weeks) while the present experiment achieved only 50% mortality (2 died and 3 were killed in anticipation of imminent death) over 47 days. Nineteen doses of 50 μg/kg were administered over 27 days and this may be significant in respect to this lower apparent toxicity.

There was, however, a marked difference between the immunised and non-immunised groups. Most importantly, there were no deaths (0% mortality) in the immunised group but 50% mortality (5 died, or killed in anticipation of death, out of 10 sheep) in the unimmunised group. The immunised group as a whole however was not free of toxic effects since some early indications of toxicity (indicated by each of the monitored parameters) were observed in 3 of the 10 sheep (details below).

(b) Food intake. Previous experience with phomopsin in sheep indicates that appetite is a sensitive indicator of lupinosis. This was again the case in this experiment, where declining appetite consistently and reliably signified the onset of clinical lupinosis. Only the BSP clearance test showed greater sensitivity, and then only in quantitative terms. Figure 6 shows the extent of this inappetence on the mean food intake for the group. Individual sheep in both groups varied widely in their response to phomopsin, although ultimately all sheep in the non-immunised group consistently left all or part of their ration. Within the immunised group, only 2 sheep (numbers 7 and 8) showed significant responses in food intake, all others consistently eating all food offered throughout. Table 3 shows the temporal relationships between the time taken for significant and consistent food refusal to become established and time to death for both groups.

TABLE 3. Time to consistent food refusal and death.

Sheep No.	Group	Time until consistent Food refusal (days)	Time to Death (Days)
1	Immunised	-	s*
2	"	-	s
3	"	-	s
4	"	-	s
5	"	-	s
6	"	-	s
7	"	29	s
8	"	21	s
9	"	-	s
10	"	-	s
11	Not immunised	19	47
12	"	21	s
13	"	22	s
14	"	22	s
15	"	21	s
16	"	20	s
17	"	13	31
18	"	7	23
19	"	12	47
20	"	20	36

* s = Survived

The extent of the effects on appetite were also variable between sheep and groups. The two affected sheep in the immunised group never became totally anorexic. Sheep 7 consistently left only small amounts of food offered, while sheep 8 left somewhat more but never more than 75% and generally less than half its ration.

(c). Liver function tests (BSP clearance).

The group means of bromosulphophthalein clearance rates during the challenge are shown in Figure 7. The unimmunised group were clearly affected to a much greater extent (mean clearance rate reduced to less than 1) than was the immunised group (minimum mean clearance rate 6.5). The control group remained within the normal range (7 to 12) during the course of the experiment. The results for individual animals (not shown) show that the slight depression in the mean BSP clearance of the immunised group was due to three individual animals, those in pens 7 and 8 (for which depression of appetite was also seen) and pen 2 (no appetite depression).

(d) Serum AST and bilirubin.

The results for both tests paralleled that of the liver function tests in that while only slight effects were seen in the immunised sheep, in unimmunised animals values for both tests became greatly elevated as the challenge progressed (Figure 8). The small elevation in the immunised group towards the end of the challenge was again due to the influence of the figures for the two most affected sheep (numbers 7 and 8).

(e) Correlation between titre and response to phomopsin administration.

Since three of the immunised sheep showed some response to phomopsin, it was possible to correlate anti-phomopsin titres with susceptibility. The BSP test results at day 28 were taken as an index of phomopsin sensitivity and plotted against the maximum titre developed by each animal (Figure 9). While only 3 points were clearly on the correlation curve, Figure 9

demonstrates that such a correlation is valid and titres above 20,000-25,000 can be considered protective in these sheep and for this challenge regimen.

EXAMPLE 7

Immunogen Formulation.

Tests have been carried out to assess the efficacy of various carrier proteins in phomopsin-macromolecule carrier conjugates in eliciting an immunogenic response in sheep.

A. Production of Conjugates.

The carrier proteins tested were fetuin, bovine serum albumin (BSA), haemocyanin, tetanus toxoid and diphtheria toxoid. For each conjugate, 15 mg of carrier protein, 19.5 mg of water soluble carbodiimide and 15 mg of phomopsin were mixed in 6.5 ml of water pH 8.0. Ammonium hydroxide vapour was introduced slowly into the reaction tube to dissolve the phomopsin. The mixture was stirred for 24 hours at room temperature followed by a further 48 hours at 4°C in the dark. After the reaction, free phomopsin was separated using a stirred ultrafiltration cell. For the phomopsin-fetuin conjugate a 10,000 MW cutoff disc membrane was used, while a 30,000 MW cutoff membrane was used for the other conjugates. After the retentates were washed 4 times with 15 ml of water at pH 8.0, the amount of phomopsin attached to the different carrier proteins was assessed as before. Results were as follows:

159 μg phomopsin:1 mg fetuin	15.9% yield*
332 μg phomopsin:1 mg bovine serum albumin	33.2% yield
347 μg phomopsin:1 mg haemocyanin	34.7% yield
173 μg phomopsin:1 mg tetanus toxoid	17.3% yield
32 μg phomopsin:1 mg diphtheria toxoid	3.2% yield

* Yield = % of available (i.e. added in reaction mixture) phomopsin actually conjugated to the carrier protein.

B. Testing of conjugates.

Five or six sheep were injected subcutaneously with each of the phomopsin:protein conjugates, the amount of phomopsin being fixed at 200 µg/dose for the primary injection and 100 µg/dose for the two boosters except for phomopsin:diphtheria conjugate. This conjugate was injected as 1.2 mg carrier/dose for primary and 0.6 mg/dose for the boosters. Priming doses were administered as 1:1 emulsions in Freund's complete adjuvant in a total of 0.8 ml and the boosters were as 1:1 emulsions in Freund's incomplete adjuvant. Sheep were bled at the time of each injection, and 3 weeks after the final booster as before.

C. Measurement of titres.

The methods used were similar to those described in Example 6 except for the following details. Positive control antiserum and serum samples from each of the test animals were diluted 1:250 in assay buffer in the second row of plates previously coated with phomopsin (50 ng/well) and washed. Serial 1:2 dilutions were made directly in the wells using an 8 channel pipette. The final volume in each well as 0.1 ml. The assay was then carried out as described previously.

Titres were determined for each sheep at each stage of the immunisation procedure.

D. RESULTS

The results given in Figure 10 (group means) show that of the 5 conjugates tested, the order of response was fetuin>haemocyanin>BSA>tetanus toxoid=diphtheria toxoid. While the fetuin conjugate generated higher

titres, the amount of phomopsin conjugated to the carrier was only about half that of the haemocyanin and BSA conjugates. This means that for a standard phomopsin dose, approximately twice the total dose of conjugate had to be administered which has implications for the economics of a commercial formulation.

It is interesting to note that the response of the sheep to the primary dose of fetuin conjugate was greater than for any of the other conjugates. Hence, it may prove in practice that a phomopsin-fetuin conjugate is also a superior immunogen in terms of achieving high titres with the minimum number of injections.

EXAMPLE 8

Enzyme Affinity Assay for Phomopsin

A. Materials and Methods.

1. Standard phomopsin solutions.

Phomopsin was extracted from lupin seed cultures of *Phomopsis leptostromiformis* and crystallised essentially according to the published method (Culvenor *et.al.*, 1977). Analysed by HPLC as a 1 mg/ml solution in methanol, this preparation was estimated as containing 87% phomopsins A and B. The 1 mg/ml solution was diluted to 10 µg/ml and subsequently to 320 ng/ml in methanol as a stock for standard curves. All methanol solutions were stored at -18°C.

2. Antisera.

Immunisation schedules and phomopsin-carrier conjugates for the sheep in which the antisera were raised have been detailed above (Example 3). CAR-HAEM antiserum was used routinely in the experiments described; the standard concentration of CAR-HAEM used for coating plates was 1/10,000.

Gamma globulins were isolated from 2 ml CAR-HAEM serum by two methods involving three (Nowotny, 1979) and one (Harlow and Lane, 1988) rounds of precipitation of IgG. The final precipitates were redissolved in phosphate buffered saline (PBS) and dialyzed against 4 x 2 litres PBS for 2 days. The contents of the bags were diluted to 1/50 of the original serum and stored at -18°C in 0.5 ml aliquots. The preparation with the three precipitation steps (Prep I) gave better recovery of antibodies and was used in the experiment described.

3. <u>HRPO conjugation of phomopsin</u>.

Three phomopsin-horse radish peroxidase (HRPO) conjugates were prepared. For each conjugate, 10 mg of HRPO and 16.5 mg of water soluble carbodiimide were mixed in 1.5 ml of H_2O at pH 8.5. In the case of conjugate No.1 (C_1), 0.788 mg of phomopsin in 78.8 µl methanol was added to the above mixture. Conjugate No. 2 (C_2) and No. 3 (C_3) were prepared by adding HRPO and carbodiimide mixture to 0.788 mg and 0.394 mg of dried phomopsin powder. Ammonium hydroxide vapour was introduced slowly into all three reaction tubes to dissolve the phomopsin. The reaction mixtures were stirred for three days at room temperature in the dark. After the reaction period, free phomopsins were separated as before and the degree of conjugation of each preparation was estimated. The phomopsin-HRPO (10 mg) was dissolved in H_2O pH 8.5 (3 ml) and stored in 0.1 ml aliquots at -80°C. For the C_1, C_2 and C_3 working stocks, each aliquot was diluted 1/20 in 40% glycerol in PBS pH 7.2, 0.01% thimerosal and stored at -18°C.

4. <u>Coating of antisera to microtitre plates</u>.

4.1 <u>Carbonate coating method</u>.

Anti-phomopsin serum CAR-HAEM was diluted 1/10,000 in carbonate coating buffer pH 9.6 (Voller *et.al.*, 1976) and dispensed into columns

2-11 of 96-well flat-bottomed polystyrene microtitre plates (Disposable Products Pty.Ltd.). The plates were dried overnight, approximately 16 hours, in the 37°C room. Thorough drying was observed to be essential for maximum absorption of antibodies to the polystyrene.

4.2 Glutaraldehyde method. (Barrett, 1977).

Glutaraldehyde (25% solution, electron microscopy grade) was diluted in 0.1 M carbonate buffer pH 9.0 and dispensed in 100 µl volumes into 96 well ELISA plates. Polymerisation was induced by heating the trays, uncovered, to 56°C for 2 hours, then cooling to room temperature for 1 hour. The plates were washed ten times with pure water. Anti-phomopsin antiserum was diluted 1/5,000 (or as indicated) in 0.1 M phosphate buffer pH 7.0 and dispensed in 100 µl volumes into columns 2-11 of the pretreated plates. The plates were dried overnight (up to 21 hours) at 37°C as above.

Coated plates were washed 4-5 times in washing solution before use. For storage, plates were sealed, with or without being washed, wrapped in plastic and frozen at -18°C.

5. Phomopsin ELISA.

The assay was performed as described in Example 4. Substrates used with H_2O_2 and HRPO were

(i) 2,2' azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)

(ii) o-Phenylenediamine (OPD)

(iii) 3,3',5,5'-tetramethylbenzidine (TMB)

all supplied by Sigma Chemical Co..

Plates were read on a Titertek Multiscan MC ELISA reader. Optical density for ABTS was read at 414 nm whereas OPD and TMB were read at 450 nm.

6. Extraction of lupin samples.

6.1 Whole seeds.

50 g of lupin seeds were soaked overnight in 250 ml of methanol:water (4:1) (extraction solvent) in a 500 ml bottle. 100 ml of the extraction solvent was then decanted off. The seed and remaining 150 ml of extraction solvent were transferred into a Servall Omni-mixer and homogenised for 3 mins. at high speed. The macerated seed was returned to the bottle and the homogeniser washed clean with the previously decanted 100 ml extraction solvent. The combined extract was stirred for 30 mins. before filtration through GF/A glass microfibre paper (Whatman). Aliquots of filtered extract were diluted 1/10 with assay buffer and the diluted extract was used in the assay at 50 µl/well.

6.2 Lupin stubble.

Large representative stubble samples were milled to about 1.0-0.5 mm. A 5.0 g subsample of the ground stubble was shaken with 100 ml extraction solvent for two hours at room temperature. The extract was filtered and diluted as above.

6.3 Flour and bran.

Representative 50 g samples were soaked overnight or shaken for 2 hours in extraction solvent, then filtered and diluted as above.

Extracts were stored at -18°C.

B. RESULTS.

1. Covalent linkage of antibodies to microtitre plates.

Coating by the carbonate method is a non-covalent process involving hydrostatic interactions between protein and plate. By creating a covalent linkage, the coating process may be made more even and leaching of

antibodies off the plastic minimised. The chosen method of covalent coating involved the polymerisation of glutaraldehyde onto the surface of the wells, generating a layer of aldehyde groups reactive with the primary amines of proteins.

Figure 11 shows a comparison between a glutaraldehyde treated plate and one simply coated by the carbonate buffer method. Pretreated plates gave higher peak optical densities (O.D.) without loss of sensitivity and very acceptable errors of ≤5% (not shown). Because the slope of the curve was steeper, it afforded more accurate readings of unknown samples within the range of the standard curve.

Barrett (1977) suggests that glutaraldehyde can be polymerised this way using a wide range of concentrations. As shown in Figure 12, glutaraldehyde concentrations from 0.1-2% were tested in order to optimise the antibody binding. For a fixed concentration of antiserum (1/5,000), 0.1% glutaraldehyde gave the highest binding without denaturation.

With glutaraldehyde coating, the concentration of anti-phomopsin serum was then adjusted (Figure 13). Even though the highest O.D. and steepest drop was seen with the 1/5,000 dilution of serum, sensitivity was best at around 1/7,000 (Table 4).

TABLE 4. Sensitivity of CAR-HAEM with aldehyde pre-treatment.

Dilution of serum	Peak O.D.	Sensitivity*
1/5,000	1.330	5-10pg
1/6,000	1.014	5-10pg
1/7,000	0.824	2.5- 5pg
1/8,000	0.397	5pg
1/9,000	0.465	5pg
1/10,000	0.472	5pg

* Sensitivity is given as the minimum amount of phomopsin standard/well that gave an O.D. ≤85% of the peak value.

2. Anti-phomopsin antibodies.

It was observed in a number of different experiments that a curve with a lower maximum O.D. would often afford greater sensitivity. In considering the effect of the concentration of antiserum added to the plates, IgG from CAR-HAEM, which had been partially purified by ammonium sulphate precipitation (Prep I), was diluted out for coating. As the concentration of antibody was reduced, the sensitivity of the assay improved until the concentration reached a level where the curves flattened out, errors increased disproportionately and sensitivity was lost. The partially purified IgG at 1/6,000 showed a definite improvement in sensitivity over the whole serum.

3. Phomopsin-HRPO conjugates.

3.1 Production of conjugates.

In making the conjugate C_1, phomopsin was dissolved initially at 10 mg/ml in methanol, whereas for C_2 and C_3, phomopsin was added dry to the reaction mixture. Solubility was ensured by

the slow introduction of NH_4OH vapour into the reaction tube. The amount of phomopsin that was conjugated to the enzyme for each preparation is given in Table 5.

TABLE 5 Phomopsin-horseradish peroxidase conjugates.

Conjugate	Initial Molar Ratio phomopsin:HRPO	% MeOH in the reaction tube	Final Ratio phomopsin:HRP
C_1	4:1	10	0.63:1
C_2	4:1	0	2.56:1
C_3	2:1	0	1.02:1

When these conjugates were tested for their ability to displace free phomopsin in the ELISA, all three demonstrated effective binding to CAR-HAEM-coated plates (Figure 14a). If the data is replotted in terms of the percent of conjugate bound with increasing free phomopsin, i.e. bound/bound at 0 pg phomopsin (Figure 14b), the biggest drop, occurring at the lowest concentration of free phomopsin was seen with C_1.

3.2 Optimal concentration of phomopsin-HRPO.

The dilution of C_1 at 1/500 had been chosen as that giving a peak O.D. of about 1.

Reduction in C_1 caused a decrease in the peak O.D. but had very little effect on the sensitivity. With all other parameters fixed as described, the optimal concentration for C_1 is around 1/700. Improvement over the dilution of 1/500, however, is marginal.

4. Substrate.

The key to detecting reactivity in any enzyme immunoassay lies in the substrate system used to indicate

the presence of enzyme. The stronger the colour produced by the chosen chromogen, the less enzyme activity that can be read, a feature critical to assay sensitivity. ABTS at pH 4 has regularly been the substrate of choice, being sensitive, very stable and its reaction with HRPO and H_2O_2 is easy to stop. Given the level of detection required of a commercially viable phomopsin ELISA, a stronger colour reaction than ABTS is needed. OPD has been reported as being more sensitive than ABTS but is very unstable in light. TMB has come into use more recently.

Both the OPD and TMB substrates were tested in the phomopsin ELISA. Figure 15a shows the O.D.s generated by OPD, used with a stopping agent, at 30 and 45 minutes of development. OPD gave a noticeably stronger colour at 45 min. than ABTS, whereas at 30 min., the difference was marginal. The TMB reaction shown in Figure 15b was stopped at 30 min. and gave a vastly superior O.D. reading to ABTS. Even though the colour intensity was much stronger, the limit of detection of the assay was not changed.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. Antibodies specific to the phomopsin mycotoxins of the fungus *Phomopsis leptostromiformis*.

2. Antibodies according to claim 1 which are polyclonal antibodies.

3. Antibodies according to claim 1 which are monoclonal antibodies.

4. A phomopsin-macromolecule carrier conjugate.

5. A conjugate according to claim 4, wherein said macromolecule carrier is selected from the group consisting of haemocyanin, fetuin, ovalbumin, bovine serum albumin, tetanus toxoid and diphtheria toxoid.

6. A conjugate according to claim 4 or claim 5 wherein said macromolecule carrier has phomopsin mycotoxins covalently linked thereto either directly or through a linking structure.

7. A method for the production of antibodies specific to the phomopsin mycotoxins of the fungus *Phomopsis leptostromiformis*, which comprises immunising an animal with a phomopsin-macromolecule carrier conjugate according to any one of claims 4 to 6, and recovering antibody-containing serum from the animal.

8. A method for the detection, and optionally quantitation, of phomopsin mycotoxins in a sample, characterised by the use of antibodies according to any one of claims 1 to 3 in an immunoassay.

9. A method according to claim 8 wherein said immunoassay is a competitive binding assay or a sandwich assay.

10. A vaccine composition for stimulating protection in animals against lupinosis, which comprises an effective amount of a phomopsin-macromolecule carrier conjugate according to any one of claims 4 to 6 as an active immunogen, together with an acceptable carrier or diluent therefor.

11. A vaccine composition according to claim 10, further comprising an adjuvant.

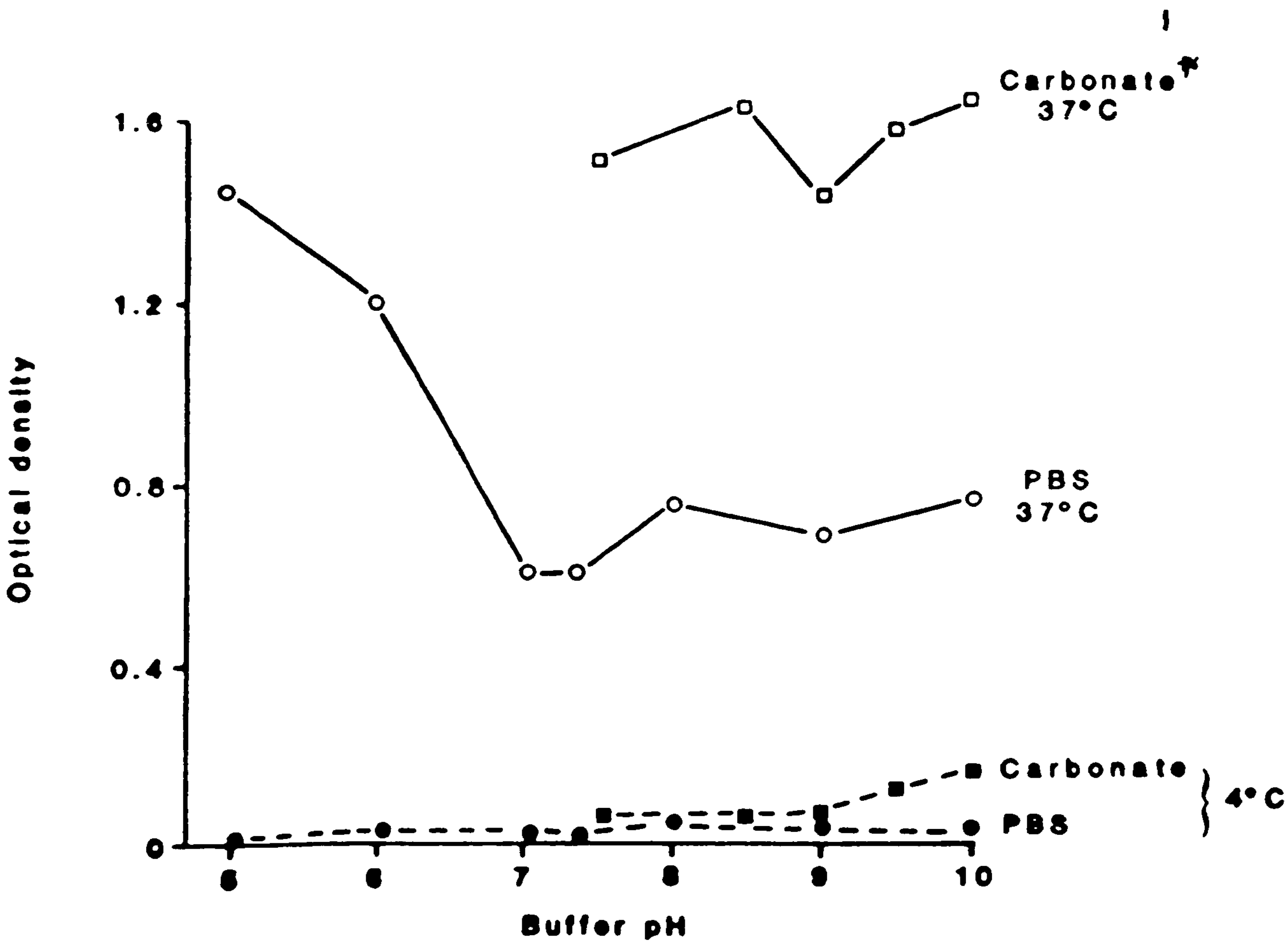
12. A method for protective immunisation of an animal against lupinosis, which comprises administration to said animal of a vaccine composition according to claim 10 or claim 11.

13. A method for the isolation of phomopsin mycotoxins from a sample, which comprises contacting said sample with antibodies according to any one of claims 1 to 3, said antibodies being immobilised on a solid substrate.

14. The steps, features, compositions and compounds refered to or indicated in the specification and/or claims of this application, individually or collectively, and any and all combinations of any two or more of said steps or features.

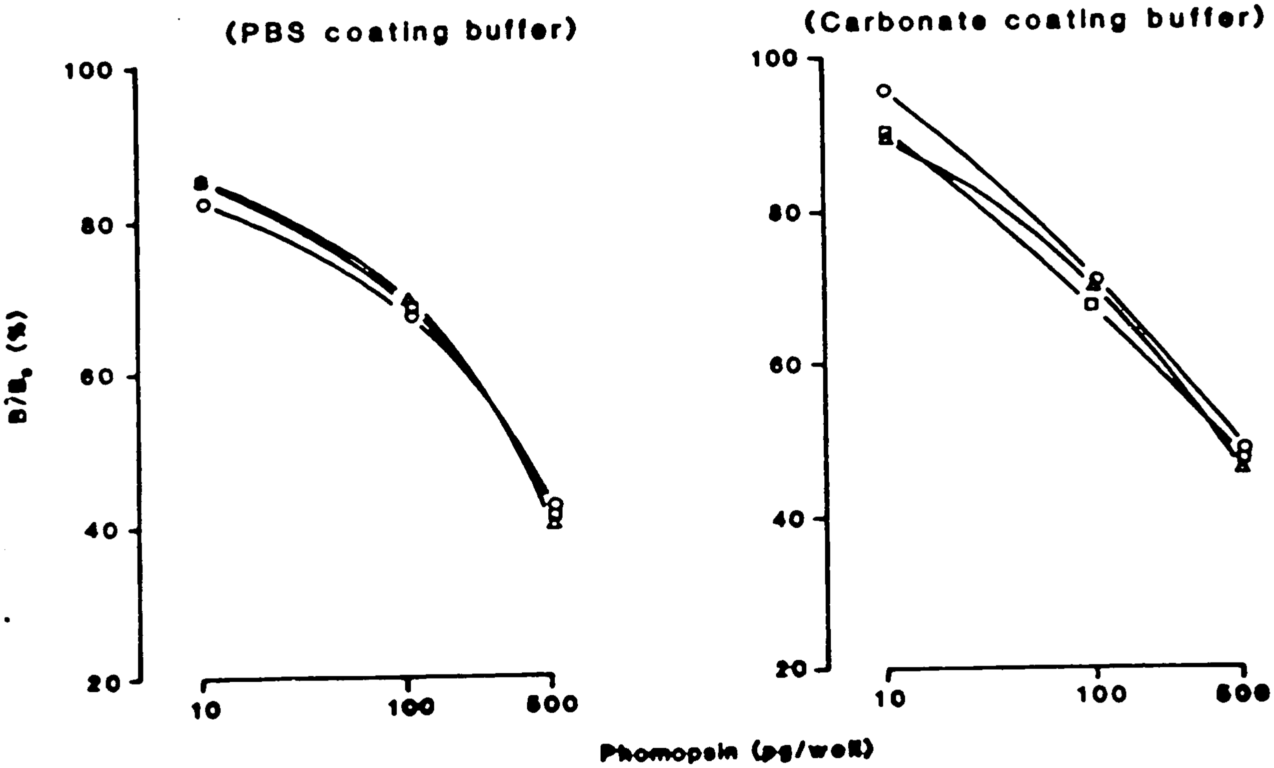
Dated this 23rd day of October, 1990,

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION,
By its Patent Attorneys,
DAVIES & COLLISON



Carbonate
37°C
1.6
1.2
0.8
0.4
0
Optical density
PBS
37°C
Carbonate
PBS
4°C
5
6
7
8
9
10
Buffer pH

FIG. 1

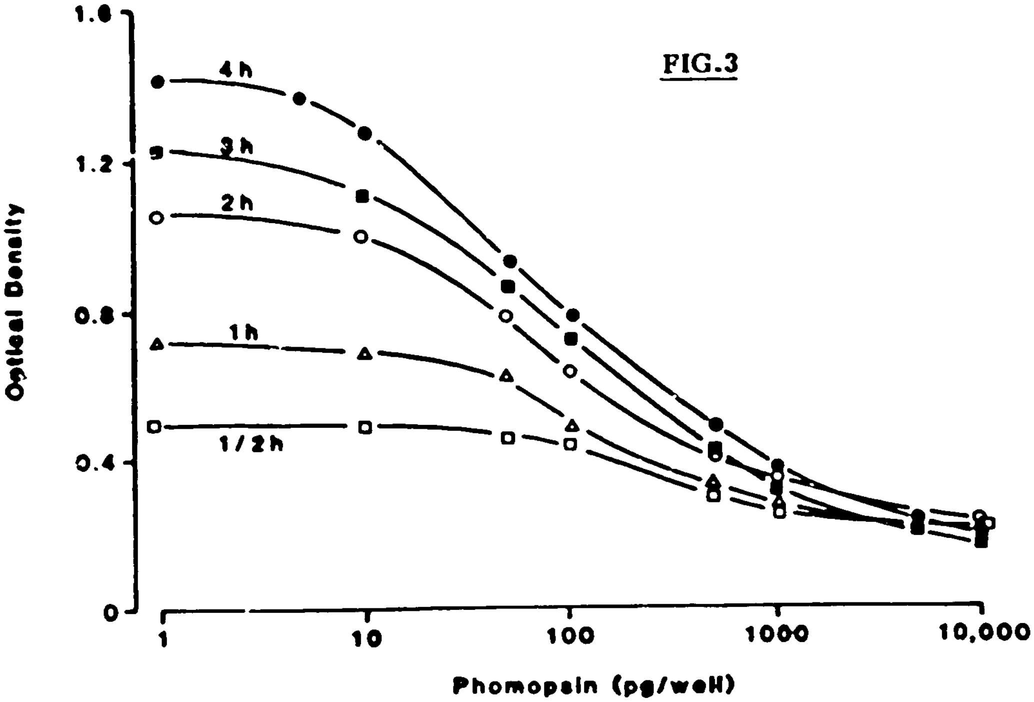


(PBS coating buffer)
(Carbonate coating buffer)
100
80
60
40
20
10
100
500
B/B$_0$ (%)
Phomopsin (pg/well)

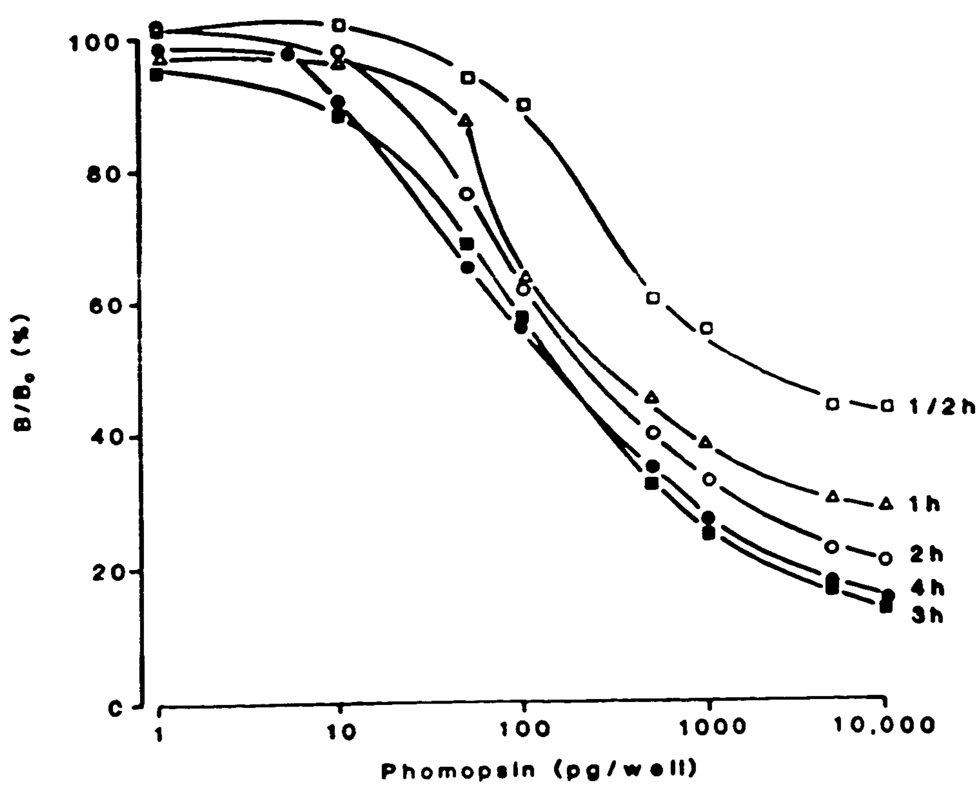
FIG.2

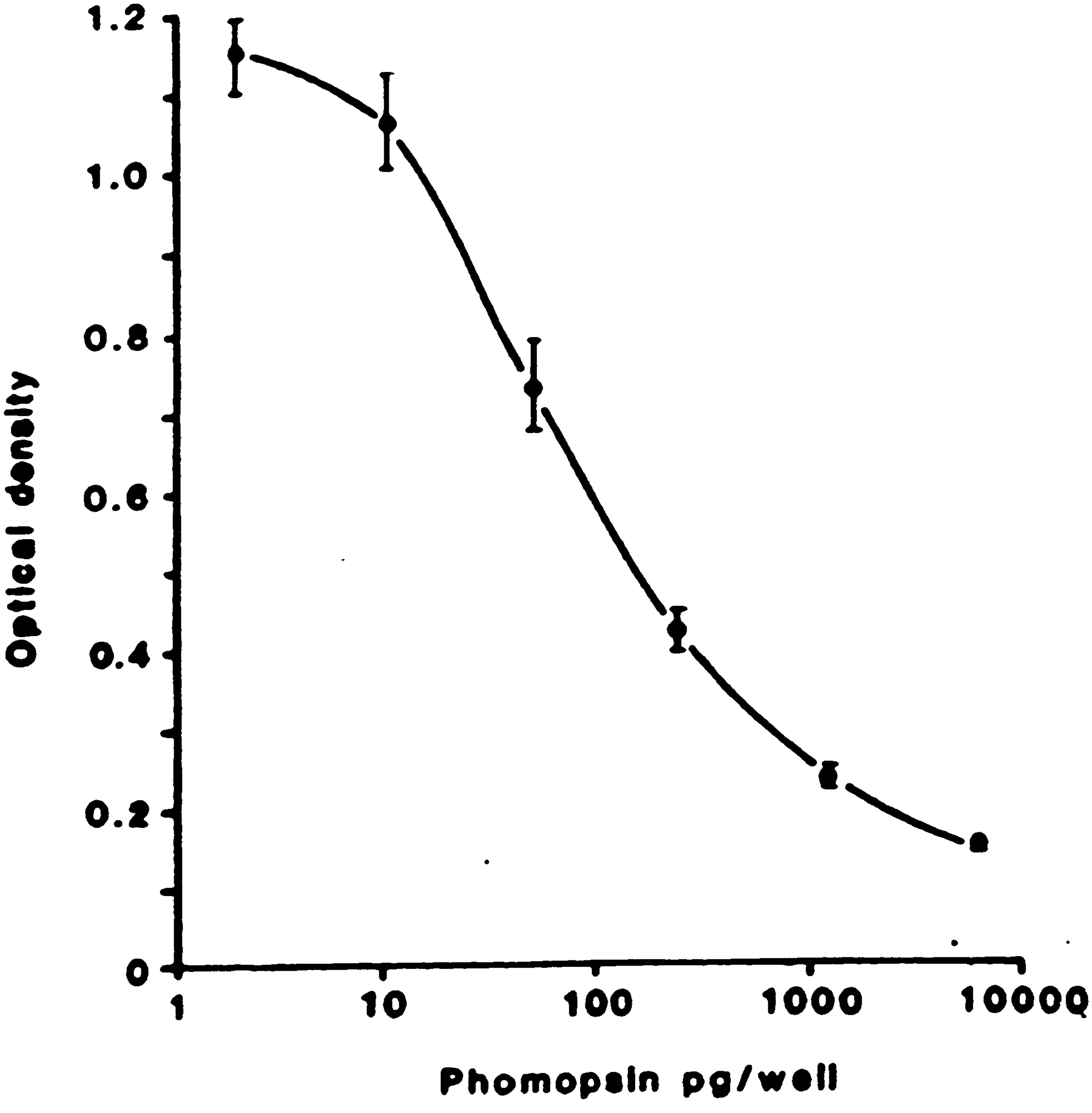
FIG.3

A.


1.6
1.2
0.8
0.4
0
Optical Density
4 h
3 h
2 h
1 h
1/2 h
1
10
100
1000
10,000
Phomopsin (pg/well)

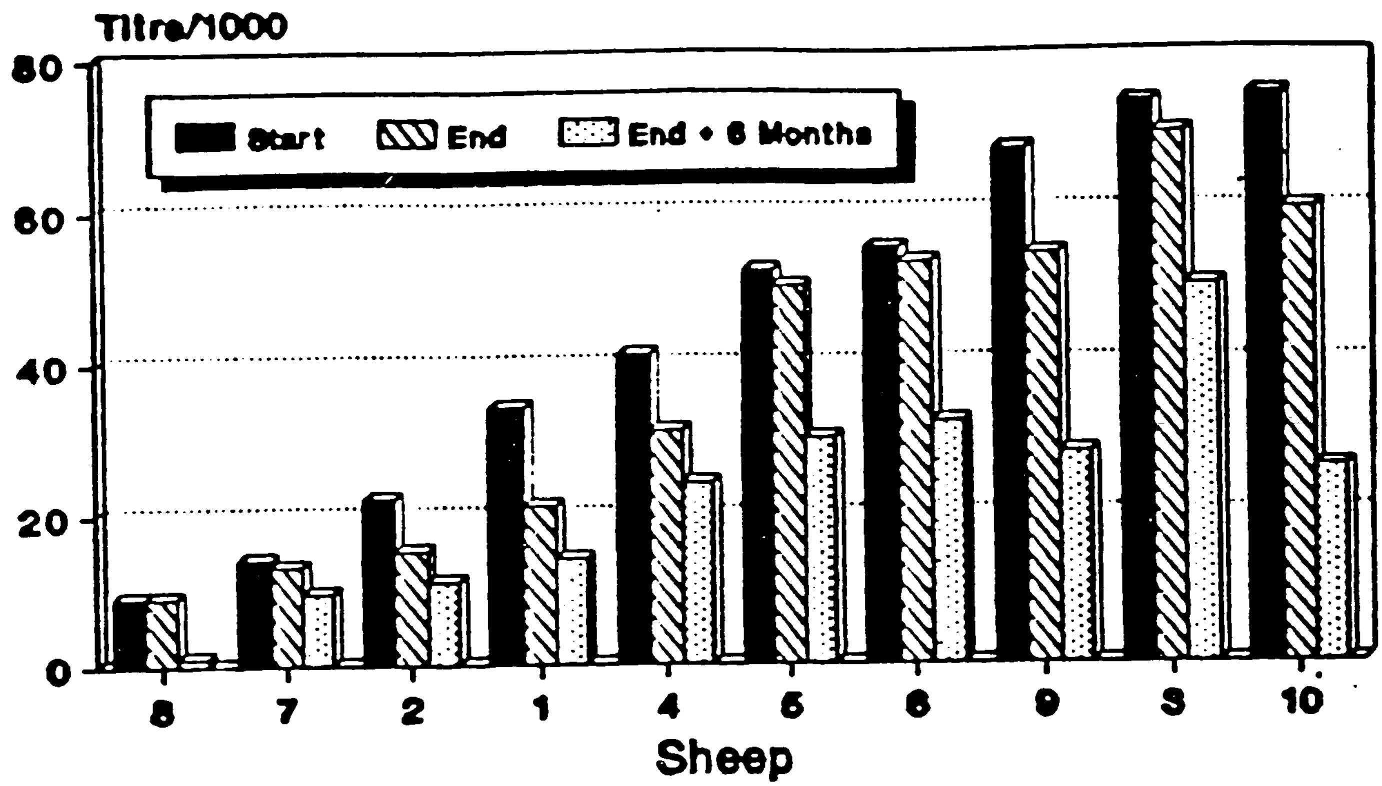
B.


100
80
60
40
20
0
B/B₀ (%)
1/2 h
1 h
2 h
4 h
3 h
1
10
100
1000
10,000
Phomopsin (pg/well)



1.2
1.0
0.8
0.6
0.4
0.2
0
Optical density
1
10
100
1000
10000
Phomopsin pg/well

FIG.4



Titre/1000
80
60
40
20
0
Start
End
End + 6 Months
8
7
2
1
4
5
6
9
3
10
Sheep

FIG.5

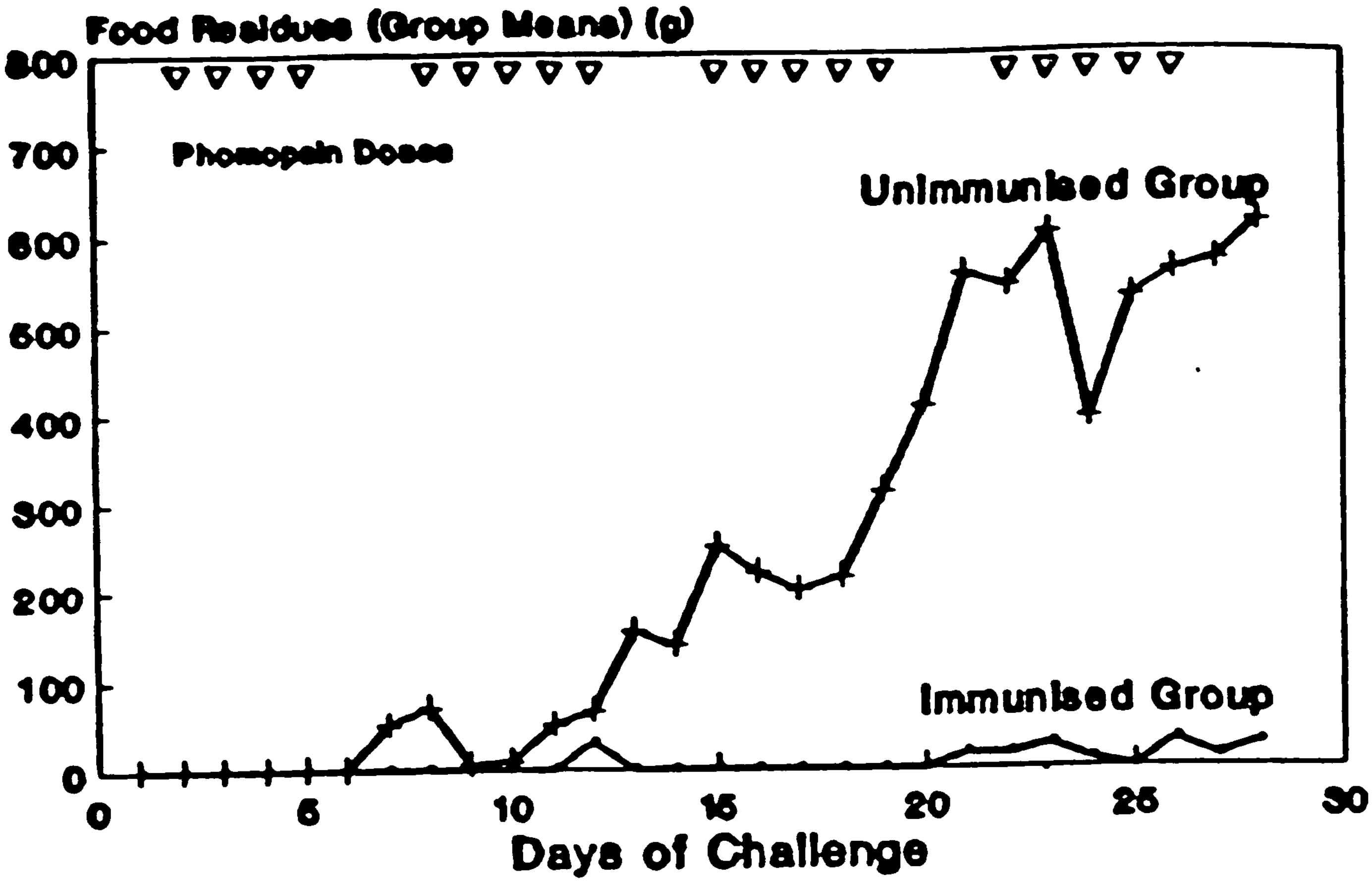

Food Residues (Group Means) (g)
800
700
600
500
400
300
200
100
0
Phomopsin Doses
Unimmunised Group
Immunised Group
0
5
10
15
20
25
30
Days of Challenge

FIG.6

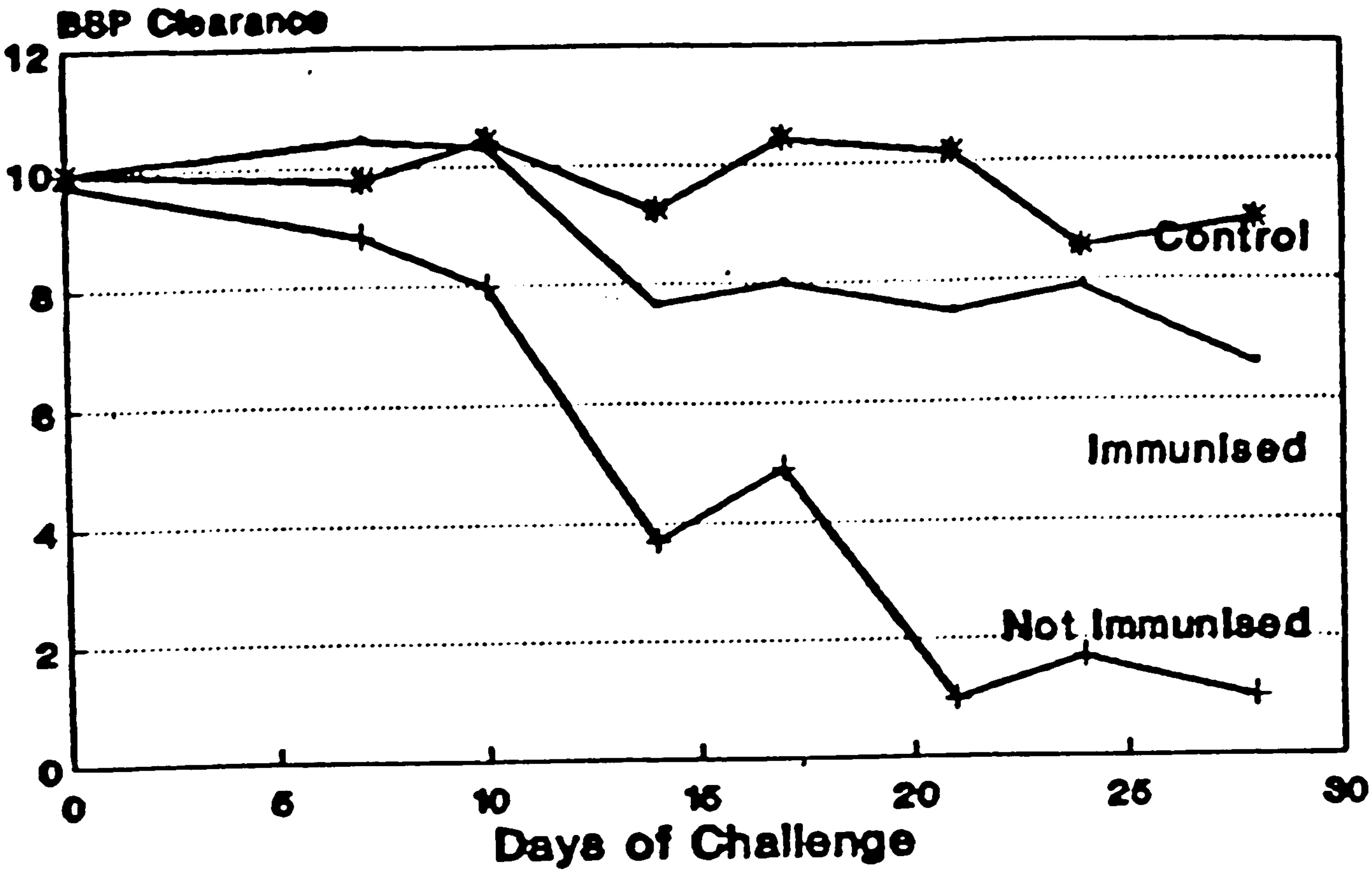
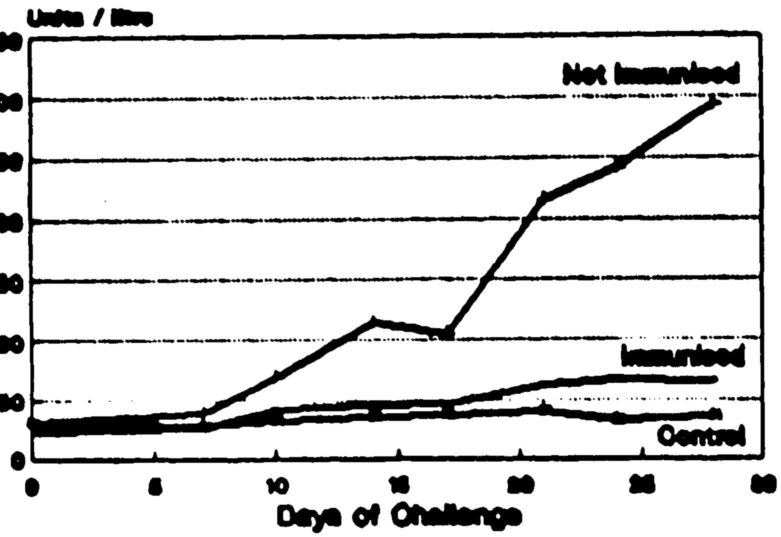

BSP Clearance
12
10
8
6
4
2
0
Control
Immunised
Not Immunised
0
5
10
15
20
25
30
Days of Challenge

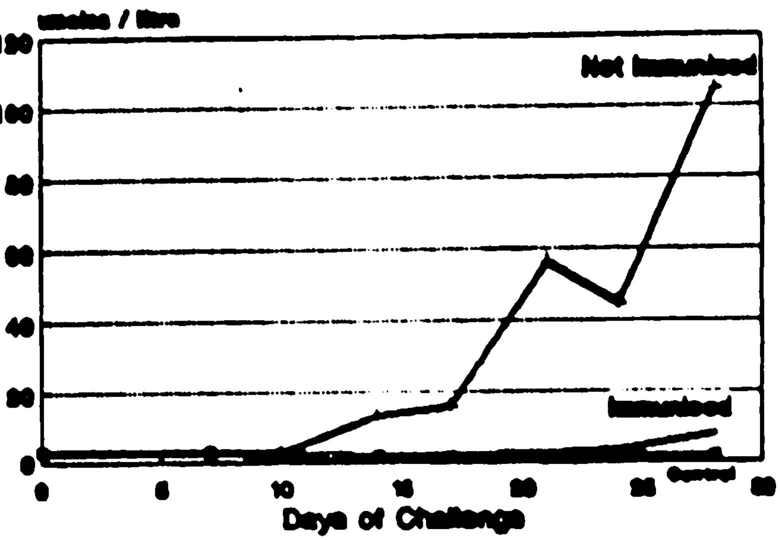
FIG.7

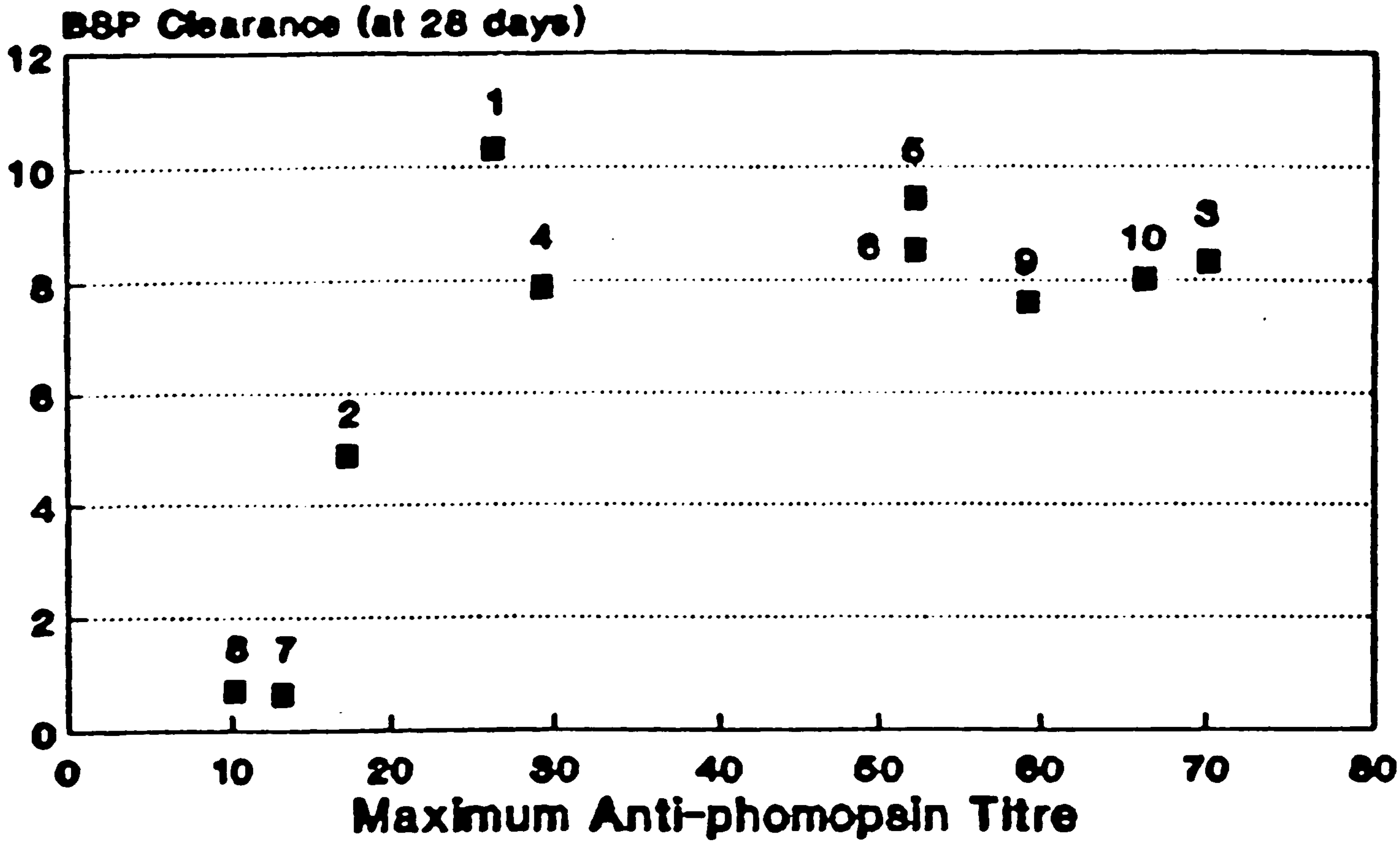
FIG.8

Serum AST values



Serum Bilirubin Values





BSP Clearance (at 28 days)
12
10
8
6
4
2
0
1
5
4
6
3
10
9
2
8
7
0
10
20
30
40
50
60
70
80
Maximum Anti-phomopsin Titre

FIG. 9

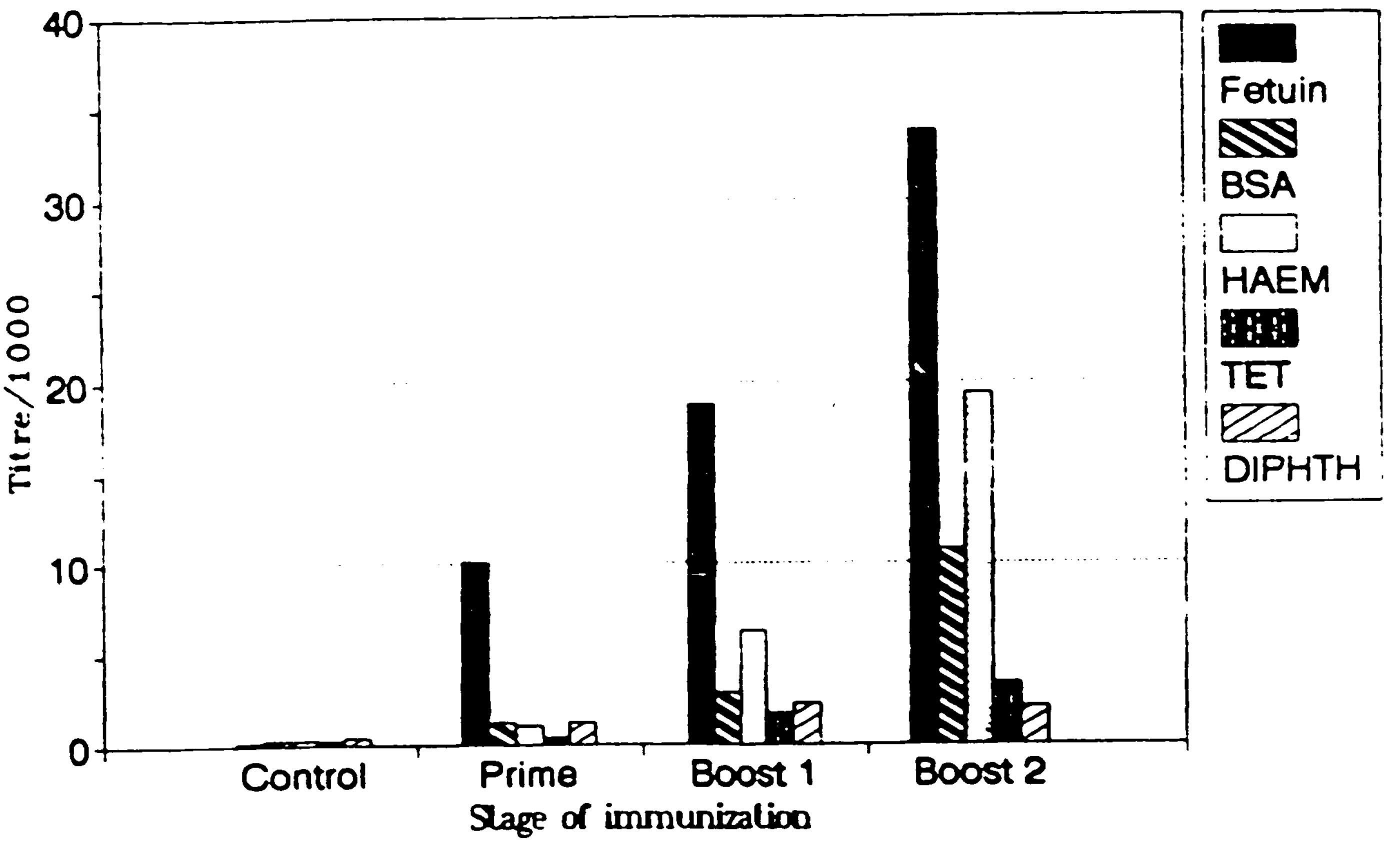
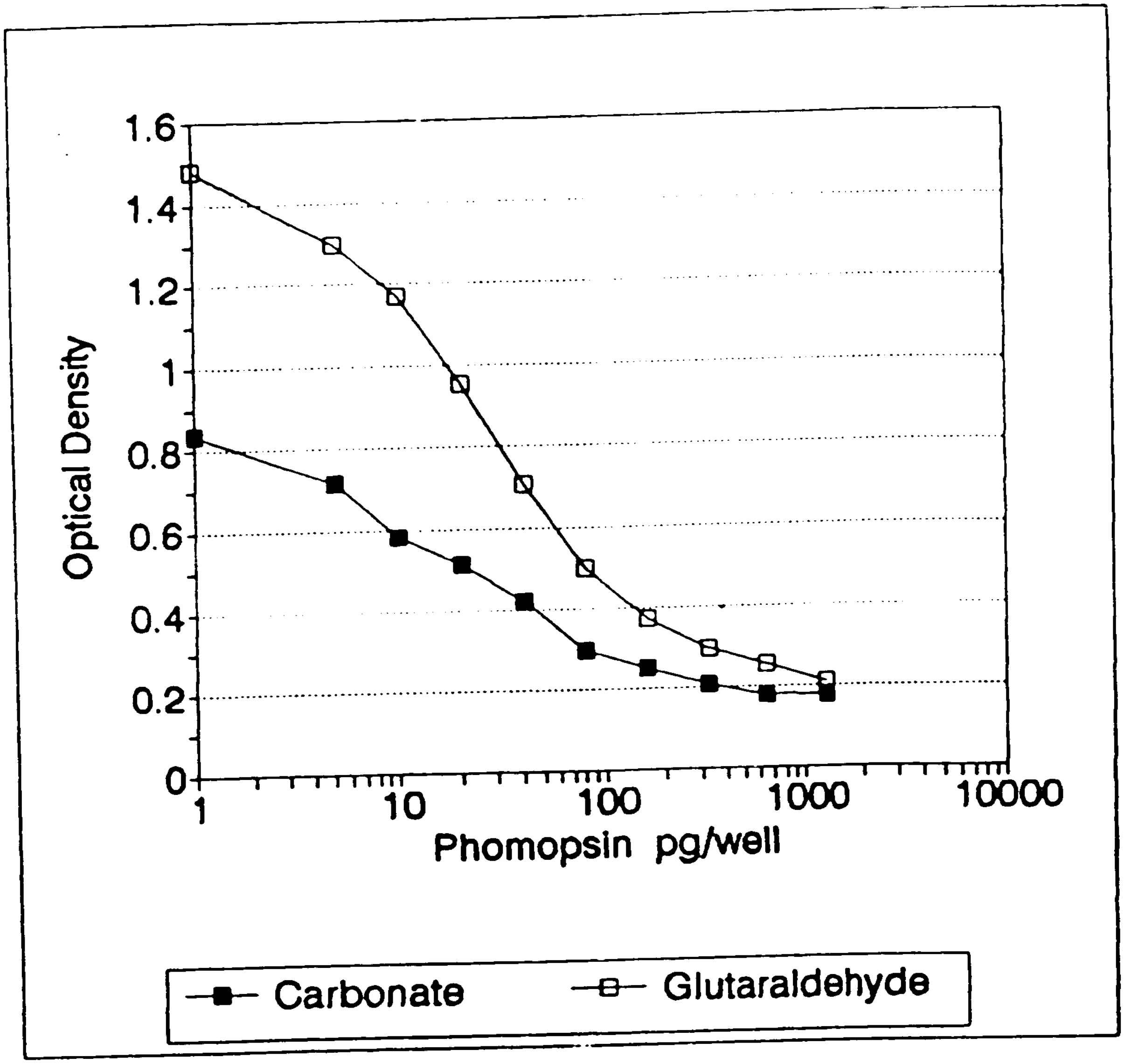
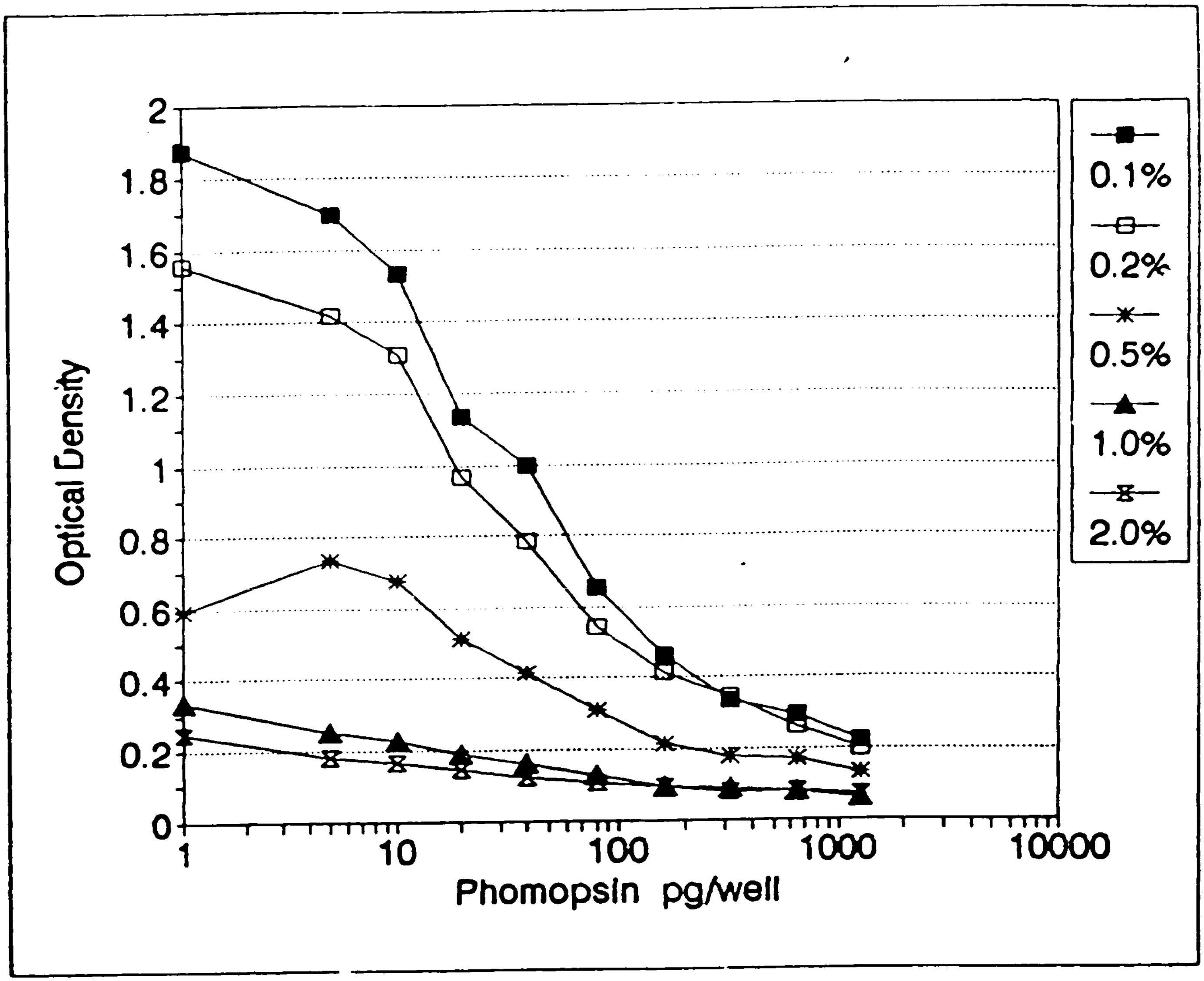

40
30
20
10
0
Titre/1000
Fetuin
BSA
HAEM
TET
DIPHTH
Control
Prime
Boost 1
Boost 2
Stage of immunization

FIG. 10



1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Optical Density
1
10
100
1000
10000
Phomopsin pg/well
Carbonate
Glutaraldehyde

FIG. 11



Optical Density
Phomopsin pg/well
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
1
10
100
1000
10000
0.1%
0.2%
0.5%
1.0%
2.0%

FIG. 12

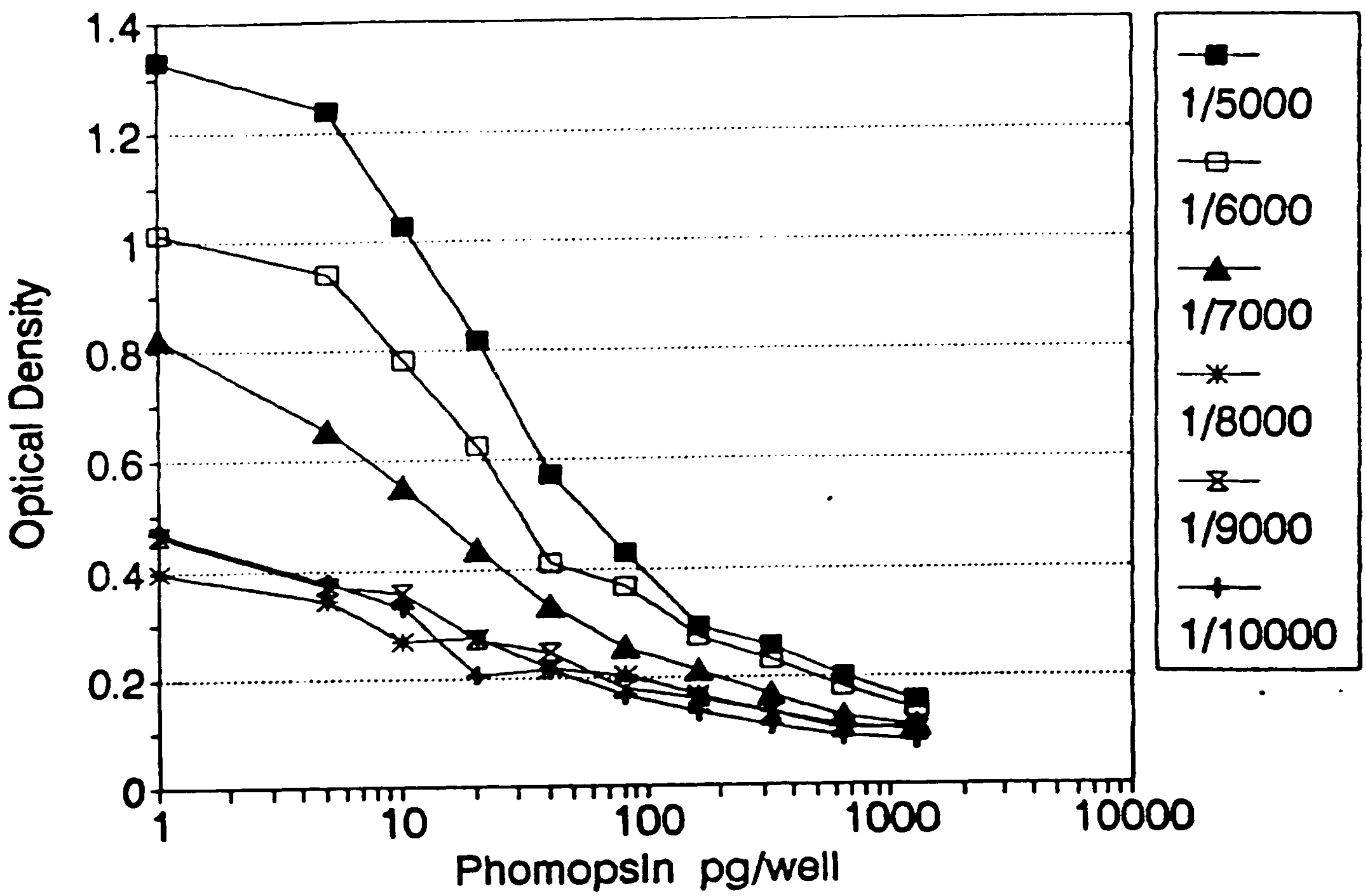
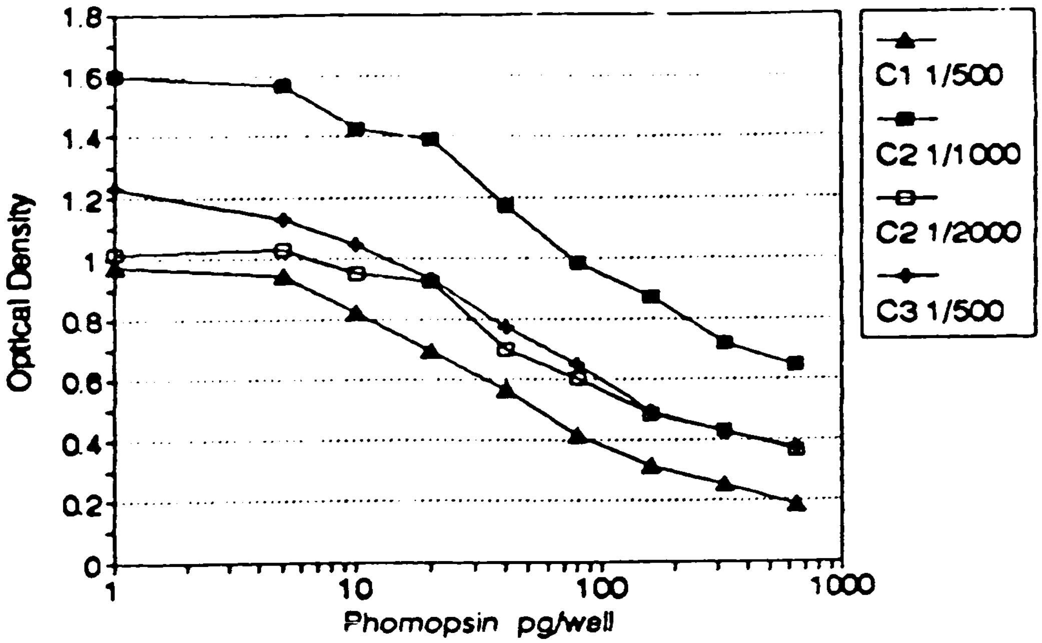

Optical Density
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
1000
10000
Phomopsin pg/well
1/5000
1/6000
1/7000
1/8000
1/9000
1/10000

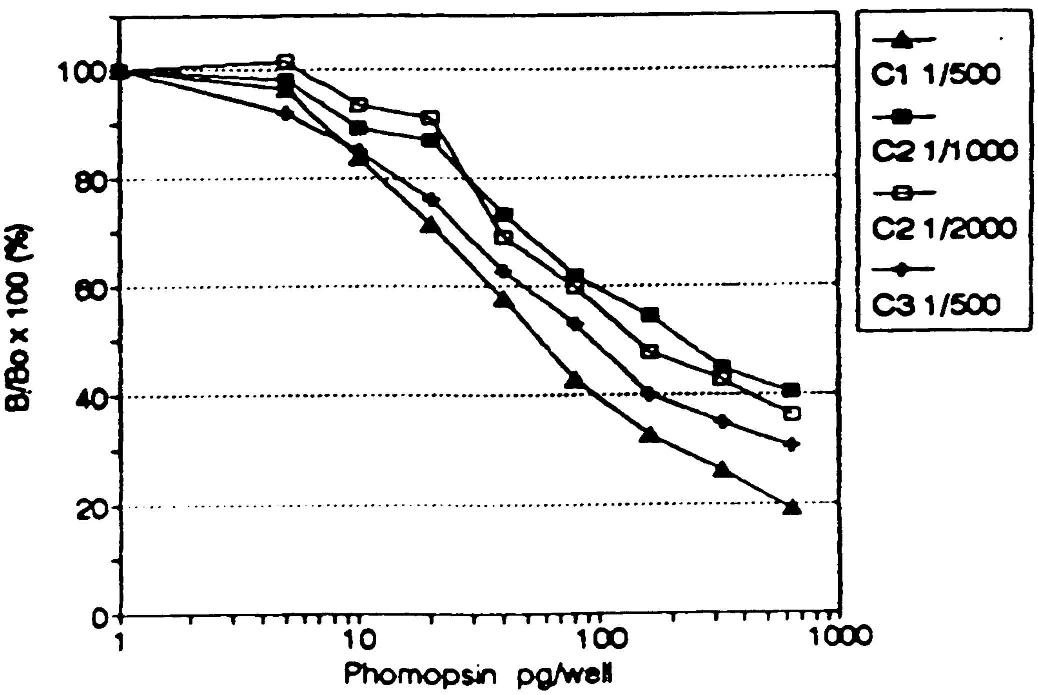
FIG. 13

(A)



1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Optical Density
1
10
100
1000
Phomopsin pg/well
C1 1/500
C2 1/1000
C2 1/2000
C3 1/500

(B)



100
80
60
40
20
0
B/Bo x 100 (%)
1
10
100
1000
Phomopsin pg/well
C1 1/500
C2 1/1000
C2 1/2000
C3 1/500

FIG. 14

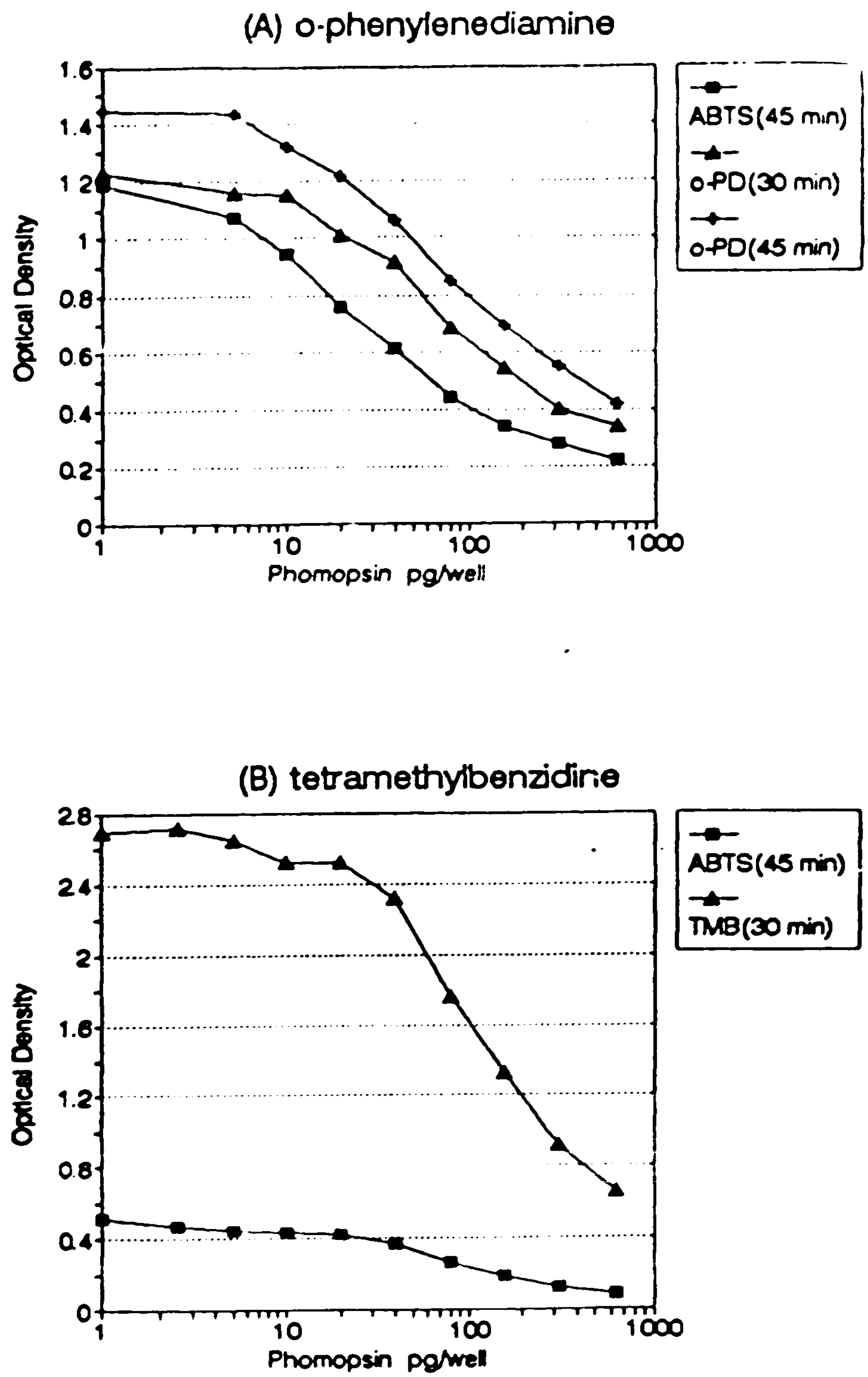

(A) o-phenylenediamine
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
1000
Optical Density
Phomopsin pg/well
ABTS(45 min)
o-PD(30 min)
o-PD(45 min)
(B) tetramethylbenzidine
2.8
2.4
2
1.6
1.2
0.8
0.4
0
1
10
100
1000
Optical Density
Phomopsin pg/well
ABTS(45 min)
TMB(30 min)

FIG. 15